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14. ABSTRACT We hypothesized that novel vitamin D analog 1α(OH)D5 (D5) will induce differentiation of dedifferentiated cells and prevent progression of malignancy in women with breast cancer. In 1999-2000, completed preclinical studies in rats showed D5 has no serious toxicity; high doses led to reversible hypercalcemic effect. In 2000-2001, we completed preclinical toxicity studies in dogs and D5 synthesis. In vitro studies suggested D5 has no effect on normal breast tissues. In 2001-2002, mechanistic studies performed and reported. In 2002-2003, in vitro studies suggested differential effect of D5 on ER+ vs. ER- cells and that VDR may partially mediate D5's action. Clinical trial protocols updated for UIC IRB and FDA. In 2003-2004, clinical protocol updated and approved by UIC IRB. Lutheran General Hospital removed from protocol. In 2004, preclinical toxicity studies completed. IND application submitted for FDA approval. FDA approved clinical protocol, withholding patient enrollment, pending clarification of drug product stability data since FDA chemists found several presumed deficiencies in study results. In 2006 pre-IND meeting, FDA approved stability study protocol. Contract was issued to complete required studies, which are underway, with results submitted to FDA. DOD has decided no further cash extension will be allowed and project is to be terminated.

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INTRODUCTION

Vitamin D and its analogs have shown potential chemopreventive and chemotherapeutic effects on various malignant tumors (1-14). The active metabolite of vitamin D3, 1,25(OH)2D3, has been shown conclusively to induce differentiation in vitro in a variety of cancer cells, including breast cancer cells (12-14). 1,25(OH)2D3 is hypercalcemic, and thus its use as a preventive and therapeutic agent is limited. Although a number of vitamin D analogs are synthesized, only limited vitamin D-related compounds have reached clinical trial. We had identified a vitamin D analog 1α-24-ethyl-cholecalciferol (1α-hydroxy vitamin D5) that showed potent growth inhibitory and cell-differentiating action in breast cancer cells. The effects of 1α(OH)D5 were extensively investigated in vitro and in vivo. We proposed to pilot 1α(OH)D5 from an experimental laboratory model to the clinical setting. The effects of 1α(OH)D5 were investigated extensively in in vitro and in vivo experimental models, and some pronounced effects of 1α(OH)D5 are summarized below.

- 1α(OH)D5 has chemopreventive action in mouse organ culture model (15).
- 1α(OH)D5 has chemopreventive action on both MNU and DMBA-induced mammary adenocarcinoma models in rats (16).
- 1α(OH)D5 has both growth inhibitory and cell-differentiating actions in human breast carcinoma cells (17,18).
- 1α(OH)D5 supplemented in the diet inhibits the in vivo growth of human breast carcinoma transplanted in athymic mice (18).
- 1α(OH)D5 is metabolized into two major metabolites (1,24 and 1,25 vitamin D5) in human breast tumors and nonmalignant breast tissues.
- The effects of 1α(OH)D5 is mediated by vitamin D receptors (VDR). 1α(OH)D5 binds to VDR with lower affinity as compared to the natural metabolite of vitamin D.
- Preclinical toxicity studies have been completed in two different species. Studies were performed in male and female rats and dogs under GLP. Adult male and female rats/beagle dogs were given 1-10 μg/kg body weight 1α(OH)D5 by gavage for 28 consecutive days. 1α(OH)D5 in rats showed no serious toxic effect. No animals died during the course of study, and no adverse treatment-related clinical signs of toxicity were observed. Increased serum calcium levels were observed in both sexes at the high dose level and in females at mid-dose levels. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at mid- and high-dose levels, and in females at all doses (19).
- The effect of 1α(OH)D5 was reversible. Within two weeks after discontinuation of the treatment, serum calcium levels and renal mineralization lesions reached the same levels as the control group (19).
- Studies were done on the in vitro effect of 1α(OH)D5 on malignant and nonmalignant tissues obtained from breast cancer patients at the time of surgery. 1α(OH)D5 had no effect on cell proliferation, cell death, or differentiation markers (casein) in nonmalignant breast tissues (epithelial cells). 1α(OH)D5 induced cell death in fibroadenomas. In malignant tumors, 1α(OH)D5 induced apoptosis (20). Preclinical

toxicity studies in dogs and rats suggested that the compound is well tolerated and causes no serious toxicity.

- We designed a clinical protocol for phase I clinical studies in breast cancer patients. The clinical protocol has been approved by both the UIC IRB as well as the FDA, and the informed consent form is approved. We have submitted an application to the FDA for approval for the initiation of a Phase I/II clinical trial of this analog in metastatic breast cancer. The FDA has requested a stability study of 1α(OH)D5 under GMP/GLP conditions.
- We gave a contract to an FDA-approved laboratory to provide us with this data. The results obtained from the stability studies indicated that the analog was stable for up to 150 days at room temperature. However, the FDA still found that stability studies were not conclusive and kept the clinical hold pending further stability studies, Additional concerns included dissolution studies, labeling errors, different stability studies for drug substance and drug product.
- Once again the effort was directed towards rectifying this situation. In order to do this, the compound was licensed to another company called "Marillion Pharmaceuticals" and the additional funding was to be provided by the Marillion Pharmaceuticals. Prior to taking this step, DOD was notified.
- This new stability study is soon to be completed and resubmitted to the FDA.
- A request was made for no cost extension to DOD, since at this time it is very likely that all the concerns have been addressed and the approval will be forthcoming.
- However the request was denied and the final report requested by the DOD.
- We expect to continue our efforts to conduct clinical trials as soon as we obtain the approval from the FDA.

BODY

Hypothesis proposed

We hypothesize that (1) 1α(OH)D5 administered to women with breast cancer will induce differentiation of dedifferentiated malignant cells and thereby prevent progression of malignancy, and (2) in women with premalignant lesions, 1α(OH)D5 will prevent dedifferentiation and thus prevent induction and/or development of breast cancer.

Technical Objectives proposed

The specific objectives of the proposed study are to:

1. Establish and evaluate biomarkers predicting 1α(OH)D5 response in malignant breast cancer and DCIS (Ductal Carcinoma in Situ).
2. Study the molecular mechanism by which 1α(OH)D5 induces differentiation/inhibits proliferation of breast cancer cells.
3. Perform (according to FDA requirement) preclinical toxicity and pharmacokinetic studies of 1α(OH)D5.

4. Initiate a phase I/II trial in advanced breast cancer patients. (During this trial, we will also obtain data on the metabolism of 1α(OH)D5 in humans.)

Successful completion of the proposed study will identify a new chemotherapeutic and possibly chemopreventive agent in breast cancer.

Results

The first three aims have been successfully completed and the results have been submitted as part of annual reports during the course of the project. The last aim has been extensively delayed due to the fact that the FDA has kept the clinical hold on the study pending clarification of the stability studies. In principle, the FDA agrees for the approval, however the stability studies were not satisfactory to the FDA chemists. In the last round of meeting with the FDA, it was found that some requested studies are cost-prohibitive, as a result we sought help of a commercial company for the support to generate data for the required stability studies (DOD was notified of these developments in a timely manner – see attachment). These studies are near completion and will once again be submitted for approval of the FDA next month. In the meantime the DOD denied our request for the no-cost extension and therefore the report falls short of completion of Aim 4 of the CTR grant. However, the study will be initiated and patient entered as soon as the FDA approval is obtained.

Effect of 1α(OH)D5 on human breast carcinoma.

We examined the effect of 1α(OH)D5 in human breast cancer tissues incubated in vitro in control and 1 μM 1α(OH)D5-containing medium. In most of the malignant breast tumors studied, the original histopathological features were preserved up to 48 hours when tissues were incubated in the control medium. Very few cells in this control tissue showed apoptotic or pyknotic changes. In contrast, cells incubated for 48 hours in medium containing 1 μM 1α(OH)D5 showed apoptotic cells. In addition, many cells at various stages of apoptosis were observed. During the current funding period, the cell proliferative index was determined by staining tissue sections with Ki67. Results indicated that there was no effect of 1α(OH)D5 on the cell proliferation of the normal breast epithelial cells in culture whereas the Ki67 staining was considerably reduced in the cancer tissues.

In previous studies, we showed that, among the various breast cell differentiation-associated biomarkers, increased α2 integrin and casein levels were the most reliable and sensitive parameters indicating response to 1α(OH)D5. We studied alpha2 integrin expression in paraffin sections of human breast carcinomas and nonmalignant breast tissues. Although the alpha2 antibody used in our studies is highly recommended for immunohistochemistry, we were unable to observe specific staining for integrin in any tissues studied. We tested several antigen retrieval systems (citrate buffer, protease digestion, trypsin digestion, SDS treatment, microwave techniques) with non-reliable results. Currently, we are analyzing alpha2 integrin expression in frozen tumor/nonmalignant breast tissues.

Nonclinical Pharmacology

The effects of 1α(OH)D5 were evaluated in a variety of experiments. Most of these results have been published (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73; Mehta RR et al. Breast Cancer Res Treat. 1993;25(1):65-71; Lazzaro G et al. Eur J Cancer. 2000 Apr;36(6):780-6). Since these results were published, a number of experiments using these four models have been performed in order to evaluate the efficacy of these agents. These include: 1) estrogen receptor (ER)-positive and ER-negative breast cancer cell lines; 2) mouse mammary gland organ culture (MMOC); 3) chemically induced rat mammary cancer; and 4) xenograft transplant models bearing human breast cancers. The following sections describe the results generated from these experiments.

Antiproliferative activity against well established breast cancer cell lines in vitro.

The effects of 1α(OH)D5 were evaluated on the proliferation of several breast cancer cell lines with known estrogen receptor (ER), progesterone receptor (PR), and vitamin D receptor (VDR) status. As shown in Table 2, these included MCF7 (ER+, PR+, VDR+), T47D (ER+, PR+, VDR+), ZR75A (ER+, PR+, VDR+), BT474 (ER+, PR+, VDR+), UISO-BCA-4 (ER-, PR-, VDR+), and MDA-MB231 (ER-, PR-, VDR-). Cells were incubated for 7 days with increasing concentrations of 1α(OH)D5 in the range of 10-9M to 10-6M. There was no effect observed at 10-9M, and no cell toxicity was observed at the highest concentration. In contrast, marked toxicity was observed when cells were incubated with 1,25(OH)2D3. These results suggest that cells required VDR in order to be responsive to either of the two vitamin D analogs. MDA-MB-231, which is characterized by the absence of ER, PR, and VDR, did not respond at all to any of the analogs. However, the presence of steroid receptors in these cells is not essential for the antiproliferative activities observed. For example, UISO-BCA-4, which lacks both ER and PR but expresses VDR, responded to both 1,25(OH)2D3 and 1α(OH)D5.

Table 1 below shows the p53, steroid, and vitamin D receptor status of the breast cancer cell lines used in these studies.

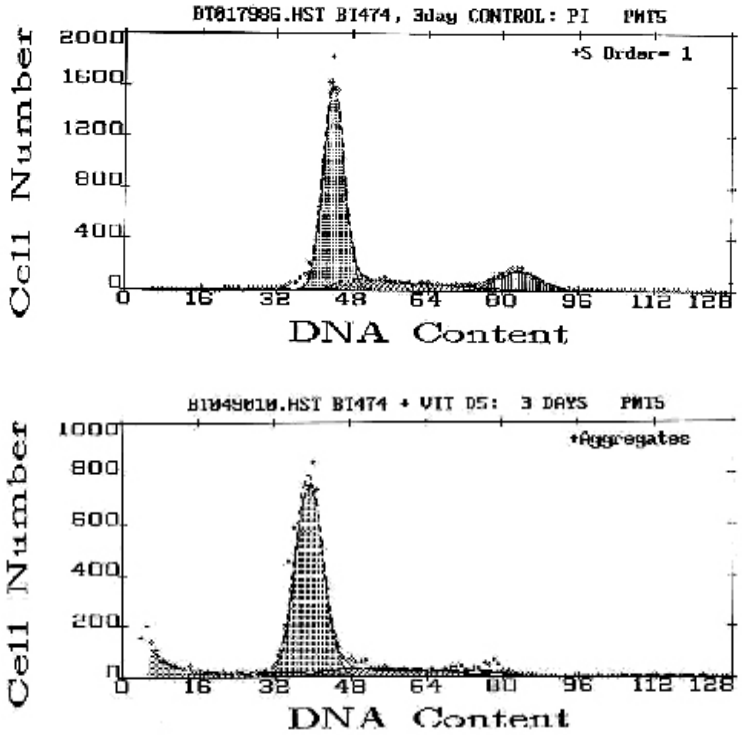
Table 1: p53, Steroid, and Vitamin D Receptor Status of Breast Cancer Cell Lines

Cell line	P53 status	ER status	PR status	Her-2 expression	VDR Status
MCF-7	wild	positive	positive	low	Positive
ZR-75-1	wild	positive	positive	medium	Positive
T-47D	mutant	positive	negative	-	Positive
BT-474	mutant	positive	positive	high	Positive
UISO-BCA-1	mutant	negative	negative	medium	Positive
MDA-MB-231	mutant	negative	negative	low	Negative
MDA-MB-468	mutant	negative	negative	Low	Negative
MDA-MB-435	mutant	negative	negative	Low	Negative
MAXF-401	mutant	negative	negative	Medium	N/A
UISO-BCA-4	mutant	negative	negative	low	Positive

These studies demonstrated that all cell lines expressing VDR (VDR-positive) are responsive to vitamin D analog and have induced cell differentiation (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73), which is characterized by increased casein and lipid expression in the cells. Furthermore, morphologically the cells exhibit signs of cell

differentiation. Most importantly, breast cancer cells that are both ER- and PR-positive in addition to being VDR positive, exhibit not only differentiation but also apoptosis. The typical effects of 1αhydroxyvitamin D5 on the cell cycle of BT474 breast cancer cell line, which is positive for ER, PR, and VDR, is shown below in Figure 1.

Figure 1: Effects of D5 on the Cell Cycle in BT474 Cells



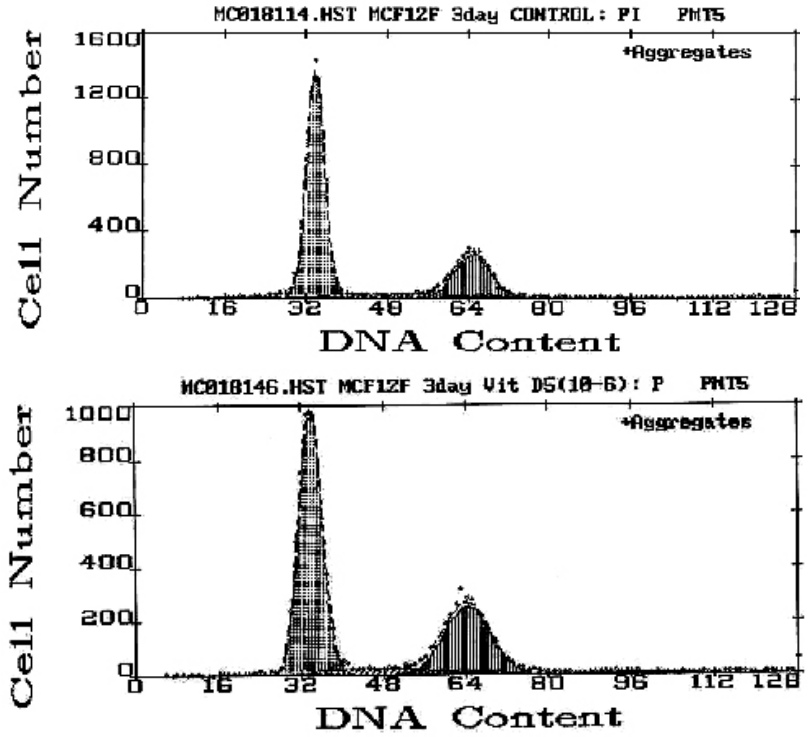
% G-1	70.3
% S	16.1
% G-2	13.6

% G-1	83.3
% S	14.9
% G-2	1.3

Selective effects of 1α-hydroxyvitamin D5 on transformed cells

A question was raised whether the effects observed against breast cancer cells can also be observed for normal breast epithelial cells. MCF12F cells are normal breast epithelial cells derived from a woman who did not have breast cancer. The cells were immortalized in culture and have been extensively used. These cells are commercially available, and this cell line was selected for the current study. Experiments were designed to compare effects of 1α(OH)D5 among these normal breast epithelial cells immortalized in culture, MCF12F, and DMBA-transformed MCF12F cells. The results are shown below in Figure 2 and Figure 3.

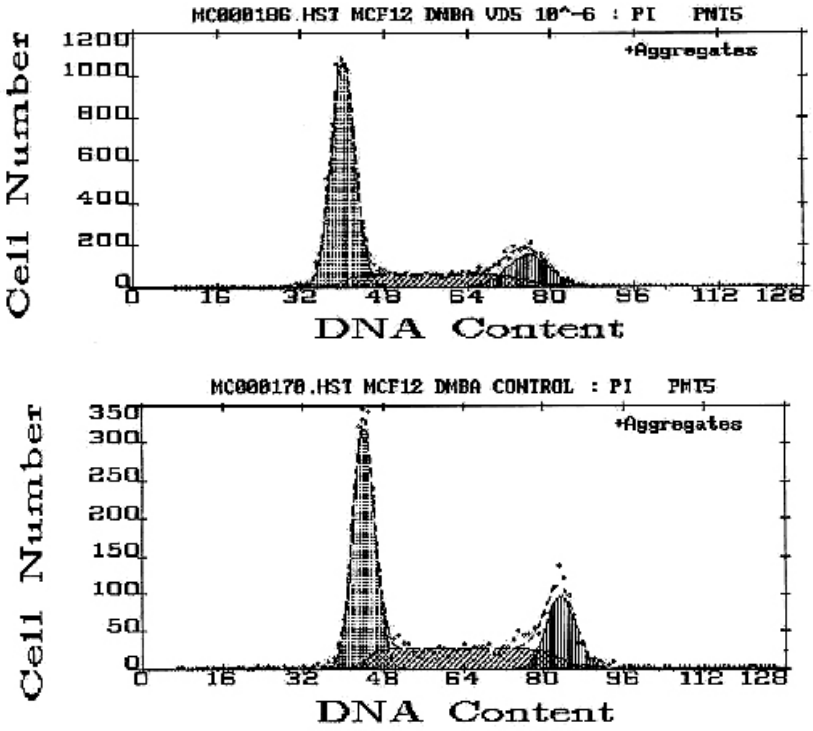
Figure 2: Effect of D5 on the Cell cycle of MCF12F Normal Human Mammary Epithelial Cells



% G-1	66.6
% S	7.6
% G-2	25.6

% G-1	65.1
% S	7.8
% G-2	27.1

Figure 3: Effect of D5 on Cell Cycle in Transformed MCF12FDMBA Cells



% G-1	49.6
% S	29.9
% G-2	20.5

% G-1	62.1
% S	21.9
% G-2	16.0

In order to further investigate this property, the MCF12F cells were transformed with chemical carcinogens N-methyl-N-nitrosourea (MNU) and 7,12 dimethylbenz(a)anthracene (DMBA). These carcinogen-treated transformed cells have altered growth rate. 1α(OH)D5 suppressed the proliferation of the carcinogen-treated cells, whereas the parent MCF12F cells did not respond (Figures 2 and 3). These results indicate that the effect of 1α(OH)D5 is selective for breast epithelial cells with altered growth characteristics as observed in breast cancer cells or carcinogen-induced transformed cells.

In summary, these results demonstrate that proliferation of BT474 cells, like the other VDR+ breast cancer cells, was inhibited by 1α(OH)D5. Moreover, cell cycle analysis showed that there was a G1 arrest in BT474 cells following exposure to 1 μM 1α(OH)D5 for 7 days. However, there was no antiproliferative effect or cell cycle arrest observed in non-transformed MCF12F cells.

Selectivity of efficacy of 1α(OH)D5 for breast cancer tissue and not normal mammary epithelium

Breast tissue samples obtained at the time of surgery of reduction mammoplasty and breast cancer samples were incubated with 1 μM 1α(OH)D5 for 72 hours. The tissues were fixed in formalin and tissue sections were prepared. The cell proliferative index was determined by staining tissue sections with Ki67. Results indicated that there was no effect of 1α(OH)D5 on the cell proliferation of the normal breast epithelial cells in culture whereas the Ki67 staining was considerably reduced in the cancer tissues (data not shown). These results are consistent with the results described in previous sections for MCF12F normal breast epithelial cells and normal mammary glands in organ cultures. These histopathologic results corroborate the findings on the growth-inhibitory effects of vitamin D5 in the in vitro studies described earlier.

Mechanism of Action of 1α(OH)D5

The effect of 1α(OH)D5 is mediated by inducing cell differentiation, and VDR is essential for the function

To examine this hypothesis, we determined the differentiating effects of 1α-hydroxyvitamin D5 in T47D human breast cancer cells. Cells incubated with either 10 or 100 nM of 1α(OH)D5 inhibited cell proliferation in a dose-dependent manner, as measured by the MTT assay. This inhibition in cell proliferation was comparable to that seen with 1,25-dihydroxyvitamin D3. Both vitamin D analogs induced cell differentiation, as determined by induction of casein expression and lipid production (Lazzaro G et al. Eur J Cancer. 2000 Apr;36(6):780-6). Induction of cell differentiation is often correlated with inhibition of cell proliferation. Casein and lipid expression are characteristics of normal lactating mammary glands. Thus, induction of these differentiation markers suggests that the cancer cells are reverting to express normal function. Since the cell-differentiating effect of vitamin D is considered to be mediated via VDR, we examined the induction of VDR mRNA using RT-PCR. The results showed that, in T47D cells, both 1,25-dihydroxyvitamin D3 and 1-Hydroxyvitamin D5 induced VDR mRNA in a dose-dependent manner. Moreover, both analogs of vitamin D up-regulated expression of vitamin D Response Element (VDRE)-VDR interaction as determined by CAT reporter assay (Lazzaro G et al. Eur J Cancer. 2000 Apr;36(6):780-6). These results

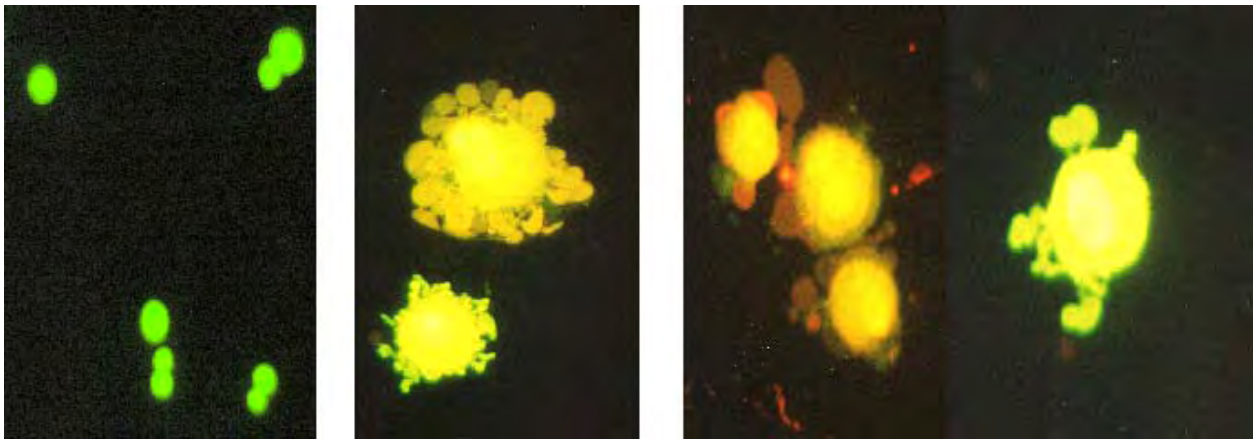
collectively indicated that 1α-Hydroxyvitamin D5 may mediate its cell-differentiating action via VDR in a manner similar to that of 1,25-dihydroxy D3

The differentiation properties of 1α(OH)D5 were further investigated in breast cancer cells. Following 10 days treatment with 1α(OH)D5 (10-7 M in UISO-BCA-4), we observed induction of intracytoplasmic casein, intracytoplasmic lipid droplets, ICAM-1, nm23, and specific biomarkers associated with breast cancer cell differentiation. 1α(OH)D5 treatment also showed induction of vitamin D receptor and TGFβ1 proteins in the cells. These results, along with the ones described in previous sections, suggest that the action of 1α(OH)D5 is mediated by VDR in breast cancer cells.

1α(OH)D5 induces apoptosis in ER+, PR+, VDR+ breast cancer cells

In ER+, PR+, and VDR+ breast cancer cells, 1α(OH)D5 induces apoptosis as well as cell differentiation, but only cell differentiation in ER-, PR-, and VDR+ breast cancer cells. We further evaluated 1α(OH)D5-induced cell apoptosis in BT474 cells. Cell cycle analysis results indicated that, in BT474, the cell growth was arrested in G1 phase. Moreover, acridine orange/ethidium bromide staining showed apoptotic fragmentation of nuclei in these cells (Figure 4).

Figure 4: Induction of Apoptosis in BT474 Cells by 1α(OH)D5



Control VD310-7 VD5 10-6

Since the only difference between these cells and BCA-4 cells was the presence of ER and PR in BT474 cells, we incubated BT474 cells with 10 nM estradiol for 5 days in steroid-stripped medium and examined estrogen-inducible expression of progesterone receptors. These cells require estradiol in the medium for cell proliferation and for the expression of estrogen-inducible genes such as progesterone receptors. The control cells expressed a higher intensity of progesterone receptors. 276 cells/351 were positively stained for PgR, whereas this PgR expression was down-regulated when the cells were incubated with 10 nM estradiol plus 1α(OH)D5.

The effect of 1α(OH)D5 was further determined by first determining the expression of D-altered genes by gene array analysis. Using the Unigene system, which examines a chip of 10,000 genes, mRNA prepared from 1α(OH)D5-treated cells was compared with that of control cells. Results showed that progesterone receptors, PS2, trefoil factor, and 24-hydroxylase were some of the genes most altered by

1α(OH)D5. These results are shown below. They clearly indicate that the effect of 1α(OH)D5 in ER+ breast cancer cells is in part mediated by down-regulating estrogen-inducible genes (See Figure 5).

Figure 5: Selected Genes from Micro-Array Analysis of D5-Treated BT474 Cells Using Human UniGene 1 (10,000 genes)

Gene Name	Differential Expression (fold)	Statistical Significance
Estrogen-inducible Genes		
Trefoil Factor 1 (pS2)	5.7 ↓	$p < 0.01$
Trefoil Factor 3 (Intestinal)	3.5 ↓	$p < 0.01$
Progesterone Receptor	3.2 ↓	$p < 0.01$
Vitamin D Regulated Genes		
Vitamin D Receptor	1.1 ↑	NS
Cytochrome P450 (Vitamin D Hydroxylase)	6.3 ↑	$p < 0.01$
Differentiation-related Genes		
Cadherin 18 type 2	3.5 ↑	$p < 0.01$
Matrix Metalloproteinase 9 (type IV Collagenase)	1.5 ↑	$p < 0.05$
Laminin Receptor 1	1.9 ↓	$p < 0.01$
Apoptosis-related Genes		
Caspase 3 (Apoptosis-related Cysteine Protease)	1.7 ↑	$p < 0.01$
Cell Growth Related Genes		
Proliferating Cell Nuclear Antigen	1.2 ↓	NS
Thymidine Kinase 2 (Mitochondrial)	1.9 ↑	$p < 0.01$

Effects of pretreatment of breast cancer cells with 1α(OH)D5 on subsequent development of tumors in mice

These studies were conducted with VDR-positive UISO-BCA-4 cells developed in our laboratory. The experiment was divided into two groups. In one group, the cells were treated with 1 μg/ml 1α(OH)D5 for 10 days, and the other group served as controls. These cells were inoculated in athymic mice and allowed to grow. All five animals in the control group developed tumors, whereas there was only a scab-like lesion in the animals where the cells were pretreated with 1 μM 1α(OH)D5 prior to inoculation. Inhibition of growth and progression of tumor in this model was attributed to 1α(OH)D5-induced differentiation of treated cells. It can be interpreted that 1α(OH)D5-induced differentiation in turn inhibited the growth and progression of breast cancer (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73).

Tissue distribution of 1α-hydroxyvitamin D5

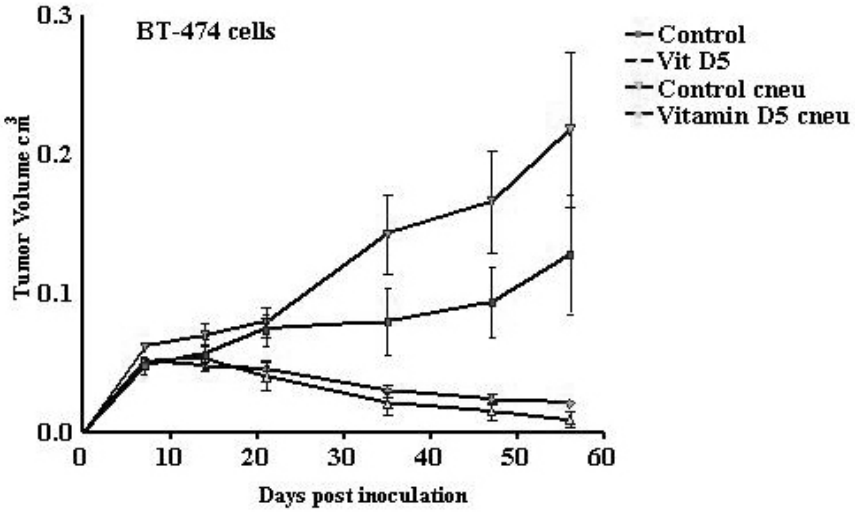
Tissue distribution studies have not been carried out in depth due to the unavailability of radioactive 1α(OH)D5. We are currently in the process of having radioactive 1α(OH)D5 synthesized. Preliminary studies were carried out to determine if

1α(OH)D5 can be recovered from plasma, liver, and mammary tumors after 2 months of feeding with 12.5 μg/kg diet 1α(OH)D5 in mice. The tissues were pulverized and extracted with methanol, and vitamin D metabolites were separated on a reversed-phase HPLC column. The HPLC profile showed the presence of 1α(OH)D5 parent compound in both mammary tissues and liver. There was no peak coeluting with 1,25 dihydroxyvitamin D3. The metabolites have not been identified due to the unavailability of standards needed for identification. However, the HPLC profile showed no peak coeluting with 1,25 dihydroxyvitamin D3.

Effects of 1α-Hydroxyvitamin D5 on the in vivo growth of breast cancer cells in athymic mice

Previously, we reported that breast cancer cells grow more efficiently in athymic mice when they are mixed 1:1 v/v of Matrigel (BD Biosciences, Palo Alto, CA) (Mehta RR et al. Breast Cancer Res Treat. 1993;25(1):65-71). Subcutaneous injection of 1-2 million cells into athymic mice results in the development of breast tumor. Histopathologically, these tumors are comparable to the parent cancers. We evaluated the effects of dietary modulation with 1α(OH)D5 on the development of breast cancers of several breast cancer cell lines. These include ZR75-1, T47 D, UISO BCA-4, and MCF-7. In most of the studies, five animals per group were used. The control animals received vehicle-containing diets, whereas the powdered diet was mixed with either 10 or 20 μg/kg of diet of 1α(OH)D5. The mice started receiving experimental diet one day after inoculation of cancer cells. The tumor size was monitored by measuring with vernier calipers. The experiment was terminated either 60 days post inoculation with tumor cells or if the tumors reached a large size (>2 cm). Results showed that 1α(OH)D5 suppressed the growth of breast cancer cells in athymic mice in most experiments except for MDA-MB-231 cells, which did not express VDR (9). An example of growth suppression of BT474 cells in athymic mice is shown in **Figure 6:** *Growth of Breast Cancer Cells in Athymic Mice*. The results collectively suggest that 1α(OH)D5 has a growth inhibitory role in VDR+ human breast cancer.

Figure 6: Growth of Breast Cancer Cells in Athymic Mice



The role VDR plays in therapeutic efficacy in human breast cancer: UISO-BCA-4 cells developed in our laboratory were divided into two groups. One group of cells was treated with 1 µg/ml 1α(OH)D5 for 10 days, and the other group served as controls. These cells were inoculated in athymic mice and allowed to grow. All five animals in the control group developed tumors, whereas there was only a scab-like lesion in the animals where the cells were pretreated with 1 µM 1α(OH)D5 prior to inoculation. These findings can be interpreted as showing that 1α(OH)D5 induced differentiation, which in turn inhibited the growth and progression of breast cancer (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73).

Toxicology

The main reason new analogs of vitamin D are being developed is to generate compounds with reduced or absent toxicity. The analog 1α(OH)D5 is one such relatively non-toxic vitamin D analog. We have completed an extensive series of preclinical toxicity studies for this vitamin D analog. In this section, we describe gross toxicity, calcemic activity in vitamin D-deficient rats, and preclinical toxicity studies under GLP in two species: rats and dogs.

Gross toxicity

Treatment of animals with vitamin D analogs often results in loss of body weight gain. This is the first noticeable toxicity. As shown below, the maximum tolerated doses were determined for athymic mice, Balb/c mice, and Sprague-Dawley rats. These doses represent concentrations at which there was no loss of body weight gains and no adverse effects on general health. Lethargy, loss of body fur, loss of weight, or loss of gain of body weight are considered as signs of gross toxicity. The animals were weighed twice a week and observed daily for lethargy and other noticeable changes.

However, no apparent side effects were noticed as a result of 1α(OH)D5 feeding in these animals.

Experiments were carried out to determine maximum tolerated dietary dose of 1α(OH)D5for rats. Sprague Dawley rats were separated into 11 groups of 10 animals each. Group 1 served as a control. Rats in other groups received either five doses (0.8, 1.6, 3.2, 6.4 and 12.8 g/kg) of 1,25(OH)2D3 or five doses (3.2, 6.4, 12.5, 25 and 50g/kg) of 1α(OH)D5for six weeks. Results showed that there was hypercalcemia and loss of body weight observed at 12.8 g/kg diet, whereas there was in fact increased body weight observed at 50g/kg of 1α(OH)D5 dose level. In a separate study there was no adverse effect of D5 on the body weight gain was observed at 100g/kg diet. Therefore, 1α(OH)D5 can be tolerated at a much higher concentration than the dihydroxy-D3 analog of vitamin D.

Measurements of calcemic activity in vitamin D-deficient rats

Male rats three weeks of age were fed diet containing 0.47g% calcium, 0.3g% phosphorus, and free of vitamin D. After three weeks of consumption of this diet, serum calcium levels were measured on selected animals. Animals exhibiting serum calcium values of less than 6.0 mg/dL were considered as vitamin D-deficient. The rats were treated intragastrically with appropriate vitamin D analog for 14 days. At the end of the study, the calcium concentrations were measured in the serum. The vehicle-treated control rats showed calcium concentrations of 5.4+ 0.3 mg/dL (mean + standard deviation). When animals were injected with 0.042 μg/kg/day of vitamin D analogs, plasma calcium concentrations of 6.0+0.6 mg /dL for 1α(OH)D5 were observed (11% increase over control, not significant from that of the control) and 8.1+ 0.1 mg/dL for 1α,25(OH)D2 D3 (50% increase over control, significant increase). A higher concentration of 0.25 μg/kg/day of 1α(OH)D5 exhibited a plasma calcium concentration of 8.1+ 0.1 mg/dL as compared to 10.1+ 1.8 for 1α(OH)2D3. Although both analogs increased serum calcium in comparison to the control samples, these results showed overall lower calcemic effects induced by 1α(OH)D5 as compared to 1α,25(OH)2D3. At higher concentrations of 1α,25(OH)2D3, there was an 87% increase in the plasma concentration of calcium as compared to the vehicle-treated rats. In contrast, when animals were injected with a higher concentration of 1α(OH)D5, there was only a 50% increase in the plasma calcium concentration as compared to controls.

Preclinical Toxicity (GLP)

Four-week oral (gavage) toxicity studies were performed on rats and dogs at the IIT Research Institute in accordance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations as set forth in the Code of Federal Regulations (21 CFR Part 58). Copies of the entire document(s) for both rats and dogs experiments are available upon request; this topic was also covered in detail in the 2002 Annual Report for this study.

Synthesis of 1α-Hydroxyvitamin D5 under GMP

As proposed in the original application the synthesis of 1α-Hydroxyvitamin D5 is being carried out by Drs. Robert Moriarty and Raju Penmasta at Conquest Inc. (formerly known as Steroids Ltd.). Dr. Moriarty has synthesized and supplied 1α-hydroxyvitamin

D5 for all our prior studies. As a part of this project, a subcontract to Dr. Moriarty is awarded for him to supply 1 gram of the compound for preclinical toxicity and 1 gram of the analog synthesized under Good Manufacturing Practice (GMP). Dr. Moriarty already synthesized and supplied 1 gram of 1α(OH)D5 for preclinical toxicity. Experiments described under preclinical toxicity used this newly synthesized compound. The synthesis of D5-analog under GMP is completed. The compound is secured in UIC Pharmacy under close control of Ms Bressler, a registered Pharmacist.

Plan for the Clinical Trial

We submitted an application to the FDA (IND #56509) for approval for the initiation of a Phase I/II clinical trial of this analog in metastatic breast cancer. The FDA has requested a stability study of 1α(OH)D5 under GMP/GLP conditions. These studies were completed in a contract laboratory. A revised application was submitted to FDA for evaluation. However the FDA also requested studies on dissolution, drug substance and drug product separately. In addition studies on the capsule-humidity were required. These studies are cost-prohibitive and therefore the analog was licensed for developing to a pharmaceutical company that can support these additional costs. These studies are now almost near completion by Marillion Pharmaceuticals under a contract to a commercial analytical company. It is expected that the application will be once again filed to FDA next month. The UIC IRB has already approved the clinical protocol. Following FDA approval, we will provide all the necessary papers to the DOD committee on human experimentation. It is expected that a "go ahead" will be received from DOD and the clinical trial will be initiated. Since these correspondence and several submissions to FDA resulted in a substantial delay in initiating clinical trial. As a result the DOD decided to terminate the project. Despite our request of no-cost extension, the DOD requested a final terminal report. As a result, we are submitting the Final Report as requested by the DOD without completing the final specific aim.

KEY RESEARCH ACCOMPLISHMENTS

Nonclinical studies:

Studies in human tumor/normal breast tissues:

The effects of in vitro 1α(OH)D5 were observed in normal breast tissues, fibroadenomas, and breast carcinomas obtained from women with confirmed diagnosis of the disease. Our results show that:

- Normal breast tissue retains the original alveolar and ductal structures when incubated in the culture medium used in this study. Breast epithelial cells appear to be normal and alive for 72 hours. All epithelial cells show VDR expression. Many appear to be proliferating, as evident from Ki-67 staining. 1α(OH)D5 (1 μM) treatment shows no toxic effect on the breast epithelial cells; all alveolar and ductal structures are preserved. 1α(OH)D5 has no effect on cell proliferation.
- Breast fibroadenomas retain normal structures in in vitro culture for 72 hours. Following incubation with 1α(OH)D5, many alveolar structures show apoptotic or degenerative epithelial cells. The cells unaffected by 1α(OH)D5 show high expression of VDR.
- Breast carcinomas treated with 1α(OH)D5 show a significant number of cells undergoing pyknosis or apoptosis.

Studies using established human breast carcinoma cell lines:

- Our results on competitive binding studies with VDR indicate that 1α(OH)D5 has relatively lower binding affinity than 1,25(OH)2D5. These results suggest that 1α(OH)D5 may possibly mediate its cell-differentiating and antiproliferative actions through VDR and also through other pathways.
- We established 4 different cell lines with different VDR and ER status. These cell lines were cloned and used to determine interaction between ER and VDR and the effect of 1α(OH)D5 on these cells. The growth inhibitory effects of 1α(OH)D5 were observed in Vitamin D receptor positive (VDR(+)) breast cancer cells, but not in highly metastatic VDR(-) breast cancer cells, such as MDA-MB-435 and MDA-MB-231, suggesting that 1α(OH)D5 action may be mediated, in part, by VDR. Breast cancer cells that were VDR+ as well as estrogen receptor positive (ER+) showed cell cycle arrest and apoptosis, while VDR+ but ER- cells (UISO-BCA-4 breast cancer cells) showed enhanced expression of various differentiation markers with 1α(OH)D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly down-regulated in ER+ BT-474 cells with 1α(OH)D5 treatment. This implies a differential effect of 1α(OH)D5 on ER+ vs. ER- cells.
- Studies on MDA-MB-231 (ER-, VDR-) cells clearly indicate that 1α(OH)D5 influences ER expression in breast cancer cells. The cells transfected with ER, VDR or ER and VDR also failed to show response to 1α(OH)D5. These results suggest that the transfection of a gene affects the expression and function of only immediate inducible gene and not the signaling cascade. ER transfection induced

progesterone receptor but did not respond to tamoxifen similarly transfection of VDR resulted in 1α(OH)D5-induced CYP24 expression but did not affect cell growth.

- We have further confirmed our previous findings that 1α(OH)D5 inhibits proliferation and induces cell differentiation markers in breast tumors (tumors obtained from patients) in vitro.

Preclinical Toxicity Studies:

- We have completed the preclinical toxicity study in male and female rats under GLP. Males and females were given 1-10 μg/kg body weight 1α(OH)D5 by oral gavage for 28 consecutive days. 1α(OH)D5 showed no serious toxic effect. No animals died during the study, and no adverse treatment-related clinical signs of toxicity were observed. No treatment-related effects on body weight, weekly or total body weight gain, or food consumption were observed during the study. Increased serum calcium levels in both sexes at the high dose level and in females at the mid dose level. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at the mid and high dose levels and in females at all dose levels. Although a no-effect level was not established in this study, the toxicological significance of microscopic lesions occurring at all dose levels was considered to be minimal because of the minimal severity of the lesions and because these lesions also occur as incidental findings in rodent studies. The effect of 1α(OH)D5 was reversible. Within two weeks after discontinuation of the treatment, serum calcium levels and renal mineralization lesions reached the same levels as the control group.
- We have completed preclinical toxicity studies in dogs under GMP. 1α(OH)D5 was tested (5-45/90 μg per kg body weight dose). The compound was given to animals daily by gavage for 28 days. At 5 μg/kg body weight dose, hypercalcemic activity was detected. The compound had some drug-related toxicity at 5 μg/kg body weight dose. All higher doses tested were toxic and hypercalcemic in dogs. Although we observed drug-related toxicity in our preclinical toxicity studies, doses tested were significantly higher than those proposed for the phase I clinical trial.

Phase I and Phase II clinical trials:

- We have obtained commercially synthesized of 1α(OH)D5 under GMP in sufficient quantity for future clinical studies. According to the requirement of FDA, we have secured the compound at the UIC pharmacy under direct control of Ms. Linda Bressler, Registered Pharmacist at UIC Hospital.
- We submitted an application to the FDA (IND #56509) for approval for the initiation of a Phase I/II clinical trial of this analog in metastatic breast cancer. The FDA requested a stability study of 1α(OH)D5 under GMP/GLP conditions. We had given a contract to an FDA-approved laboratory to provide us with this data. The resubmission to FDA once again came back unapproved with additional requirements. These included determination of toxicity in both drug substance as

well as drug product (capsules), dissolution studies, humidity studies, denaturation studies on GMP material. We gave another contract to a different commercial analytical laboratory that specializes in these studies (These studies are conducted with the funding from the Marillion Pharmaceutical Co.). In addition that required synthesis of additional 1α(OH)D5 under identical condition by the same company. This also is now completed and new GMP-D5 is received (supported by Marillion Pharmaceuticals). It appears that new revised application will be submitted to FDA next month. The trial should begin soon after that. The UIC IRB has already approved the clinical protocol and informed consent form.

Tasks originally proposed but not completed in the proposed time line and are currently under investigation:

1. Initiation of a phase I clinical trial of 1α(OH)D5 was originally proposed to initiate by 2001; however, due to FDA and UIC IRB hold and failure to obtain timely approval from the FDA after 3 submissions delayed this initiation. The UIC IRB approval has been obtained. The FDA wishes further studies on the stability of Vitamin D5. Vitamin D analog is synthesized under GMP conditions, twice, and is available in sufficient quantity to conduct the proposed trial. As soon as the results are available they will be sent to the FDA and receive approval for the trial.
2. As soon as FDA approval is received, we plan to initiate a phase I trial. The vitamin D analog is synthesized under GMP regulations and is available for the clinical use.

REPORTABLE OUTCOMES

Publications:

1. Lazzaro G., Agadir A., Qing W., Poria M., Mehta R.R., Moriarty R.M., Zhang X., Mehta R.G. Induction of differentiation by 1α(OH)D5 in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur. J. Cancer 2000; 36: 780-786.

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See Appendix 3 for copies of all of above publications except #16, which will be published by Carcinogenesis this year.

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CONCLUSIONS

We have completed the first three aims and tasks originally proposed in the application. We have performed studies in cell lines and have completed detailed preclinical toxicity studies in dogs and rats under GLP. We have completed synthesis of 1α(OH)D5 under GMP for future clinical trial. In vitro studies in clinical specimens obtained from women suggest that 1α(OH)D5 has no effect on normal breast tissues; it inhibits cell proliferation in tumor cells. This implies that it has no bad effects on normal breast tissues but does inhibit cancer growth. 1α(OH)D5 or its active metabolite possibly interacts with estrogen receptor. We submitted our IND application to the FDA, however it was returned twice for additional studies. We are in the process to complete this extensive list of requirements and plan to resubmit it next month for the approval. The UIC IRB has approved the clinical protocol and informed consent form for the Phase I clinical trial. However from the DOD point of view, this delay was unacceptable and our request for no-cost extension was rejected resulting in this final report without completion of the fourth specific aim and task.

We expect that this analog of vitamin D will be tolerated by the patients in Phase I trial and hopefully will prove successful for breast cancer patients .

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Appendices

Appendix 1: DoD Correspondence

From: Tapas K. Das Gupta [mailto:tkdg@uic.edu]
Sent: Wednesday, August 02, 2006 5:42 PM
To: Christian, Carole B Dr USAMRMC
Cc: Scassero, Shannyn M Ms USAMRAA; gislason@uic.edu; mdicig@uic.edu; tanya@uic.edu
Subject: DAMD-17-99-1-9233

August 2, 2006

Carol B. Christian, Ph.D.
Grants Manager
Congressionally Directed Medical Research Programs

Dear Dr. Christian:

Re: DAMD-17-99-1-9233

I have encountered an almost insurmountable problem since my last email to Shannyn on 7/05/06. Unfortunately, the company contracted to perform stability studies has not been able to produce data acceptable to FDA chemists. It appears that to contract out to another company in the US will require an additional $100,000. If I do this, I shall be left with only $100,000, which is not sufficient to initiate a Phase I/II trial as stipulated in the grant.

There is an additional complication. Professor Moriarity (Emeritus Professor of Chemistry, University of Illinois at Chicago), in whose laboratory Vitamin D5 analog was synthesized, along with four other chemists own the patent for Vitamin D5. (UIC does not own the patent.) During the course of the grant, his group provided all the required Vitamin D5 to perform all the studies outlined in the grant proposal as well as provided us with clinical grade Vitamin D5 for the projected Phase I/II clinical trial.

Unknown to me, Professor Moriarity s group has given the exclusive license to perform Phase II/III clinical trials and commercialize the product to Marillion Pharmaceuticals, Inc. (CEO, Dr. Zahed Subhan).

Dr. Subhan has just contacted me suggesting that I transfer the IND to the company, and his company after receiving FDA permission will get all the stability studies done and will partner in Phase I/II trial as outlined in the grant proposal, apparently with additional support.

I have told him that I have no authority to pursue any negotiation with him or anybody else without express permission from both the DoD as well as the Office of the Vice Chancellor for Research of the University of Illinois at Chicago (OVCR). However, I have also told him that I shall bring this up for your and OVCR s consideration. I think the idea behind this is similar to the RAID program of NCI. I am currently conducting a Phase I trial under the aegis of RAID.

I shall wait to hear from you before I communicate with Marillion Pharmaceuticals.

Thank you for your patience and consideration.

Sincerely,
Tapas K. Das Gupta, M.D., Ph.D., D.Sc.

Subject: RE: DAMD-17-99-1-9233
Date: Wed, 24 Jan 2007 10:03:52 -0500
From: "Scassero, Shannyn M Ms USAMRAA" <shannyn.scassero@us.army.mil>
To: "Tapas K. Das Gupta" <tkdg@uic.edu>
Cc: <gislason@uic.edu>, <mdicig@uic.edu>, <tanya@uic.edu>,
"Christian, Carole B Dr USAMRMC" <carole.christian@us.army.mil>,
"Fisher, Pam L Ms USAMRAA" <pamela.l.fisher@us.army.mil>

Dr. Das Gupta,

The Government herein denies your request as outlined in the e-mail below. Based on the SOW, the reports provided to date and the issues discussed in the 2 AUG 06 e-mail, this award will be allowed to expire 31 MAR 07. Your institution is responsible for closing out this award in the manner instructed by your award. This includes submission of a Final SF272 (due no later than 15 APR 07), a Final Scientific Report (due no later than 30 APR 07), A Patent Report (due no later than 30 APR 07), a Cummulative listing of only the nonexpendable personal property acquired with award funds for which title has not been vested to your institution (due no later than 30 APR 07) and a check made out to the U. S. Treasury for all unexpended funds received under this award.

Currently, your award is non-compliant as I have not received the last SF272 for the period 1 OCT 06 through 31 DEC 06, which was due 15 JAN 07. Both your you and your institution have been placed on a non-compliant list until receipt of the afore mentioned report. Please understand that such a listing means future funds may not be awarded to your or institution until all issues are rectified. Request this report be submitted immediately.

If you have any questions, please contact me for assistance.

Sincerely,
Shannyn M. Scassero
Contract Specialist
USAMRAA
(P) 301-619-2640

Date: Thu, 01 Feb 2007 12:35:11 -0600
To: "Scassero, Shannyn M Ms USAMRAA" <shannyn.scassero@us.army.mil>
From: "Tapas K. Das Gupta" <tkdg@uic.edu>
Subject: RE: DAMD-17-99-1-9233
Cc: bbarrie@uic.edu,dale.case@amedd.army.mil

Dear Ms. Scassero,

I am indeed sorry to hear that DoD is denying my request of 8/2/06.During the intervening 6 months considerable progress has been made in the FDA's requirements pertaining to chemistry and stability studies. Below is attached the letter I have just received from Marillion. Based on the information which has been provided,after the submission of the response by 3/14/07, it is most likely that by the end of April the clinical trial can be started.Under these circumstances, I would appeal to the Government to allow me an extension without cost for 1 year.

I recognize your patience is running out, however, I have no control over FDA's working and their policy decisions! Thus I am making this appeal, my colleagues and I have worked quite hard to bring this VitaminD5 analog to a clinical trial and we sincerely hope that you will see fit to let us finish this with the blessing of DoD. Thanks TKDG.

Appendix 2: Protocol for Clinical Trial

A Phase I/II Trial of 1αhydroxyvitamin D_5 in the Treatment of Metastatic Breast Cancer

Co-Investigators

Tapas K. Das Gupta, M.D., Ph.D., D.Sc.

George I. Salti, M.D.

Sub-Investigators

Cathleen Schaeffer, R.N., B.S.N., O.C.N.

Linda Bressler, Pharm.D.

Protocol No.: UISO – D5 – 001 – 03

Protocol Synopsis

Title:	A Phase I/II Trial of 1αhydroxyvitamin D_5 in the Treatment of Metastatic Breast Cancer
Objective:	To evaluate the safety and efficacy of 1αhydroxyvitamin D_5 (Vitamin D5) in the treatment of patients with metastatic breast cancer
Population:	Patients with metastatic breast cancer
Sample Size:	42 patients
Dosage/Treatment:	Based on completed preliminary studies (see attached investigator's brochure), the first six (6) patients will receive a single daily oral dose of 1αhydroxyvitamin D_5 starting at 5 μg gelatin capsule. If there is no toxicity (see Section 8.0), the next 6 patients will be treated similarly with 10 μg daily. The dose will be escalated (in 5 μg increments) up to a maximum of 35 μg daily.
Duration:	Treatment will be continued for three months (12 weeks) and/or disease progression, though blood tests will continue monthly for 28 weeks and then every two months for an additional six (6) months of follow-up.
Endpoints:	Safety – Clinical and laboratory adverse reactions will be closely monitored by periodic physical and laboratory examination. Grade 3 nonhematologic or grade 4 hematologic toxicity will define the maximum tolerated dose (MTD) (Appendices 1 and 2). Evidence of hypercalcemia will be the primary determining factor in dose escalation. Efficacy - Clinical response as measured by decrease in measurable disease determined by physical examination, radiographic studies, and/or nuclear medicine scans.
Co-Investigators:	Tapas K. Das Gupta, M.D., Ph.D., D.Sc. George I. Salti, M.D.
Sub-Investigators:	 Cathleen Schaeffer, R.N., B.S.N., O.C.N. Linda Bressler, Pharm.D.

1.0 OBJECTIVES

To evaluate the safety and chemotherapeutic efficacy of 1αhydroxyvitamin D_5 ($1\alpha(OH)D_5$) in patients with metastatic breast cancer.

2.0 BACKGROUND AND RATIONALE

2.1 Disease background

Although a number of patients with localized breast cancer can be adequately treated with surgery and radiation therapy, for the vast majority of patients additional use of conventional chemotherapeutic agents and hormonal therapy is necessary. While initially responsive to various cytotoxic and hormonal modalities, most breast cancers ultimately acquire resistance to current systemic therapies. Thus, the development of effective new therapeutic modalities is critical. Recently, several vitamins and vitamin analogs have been the foci of investigation as therapeutic agents for various malignancies. Among various vitamins, vitamin A[1,2,3,4,5,6] and vitamin D[7,8,9,10,11,12] have shown the most promising results. The active metabolite of vitamin D_3, 1α,25 dihydroxy D_3 ($1\alpha,25(OH)2D_3$), has been conclusively shown to induce differentiation *in vitro* in a variety of cancers, including breast cancer.[13,14,15,16,17,18,19,20] Similarly, physiologic levels of 1α,25 dihydroxyvitamin D_2, which has less calcemic activity, have been shown to induce differentiation in androgen-independent prostate cancer. However, most of the D group of vitamins and closely related metabolites are limited in their translational use due to their hypercalcemic activity. Numerous analogs of vitamin D_3 have been synthesized in search of a non-calcemic or relatively less calcemic vitamin D with similar antiproliferative effects.[21,22,23,24,25,26,27]

In the present study, we aim to evaluate the chemotherapeutic potential of another analog: $1\alpha(OH)D_5$. Using breast tumors with different histologic subtypes and with different molecular and biological characteristics, the effects of $1\alpha(OH)D_5$ (D_5) on breast cancer cell growth and differentiation both in vitro and in the athymic mouse model have been evaluated (for details, see investigator's brochure). Based on these laboratory studies, the present phase I/II clinical trial will be initiated, to determine the dose tolerance and efficacy of $1\alpha(OH)D_5$ in advanced breast cancer patients.

2.2 Vitamin D_5 Analog Background

$1\alpha(OH)D_5$, a new non-calcemic vitamin D analog synthesized by our group, has shown potent activity against human breast cancer in xenograft models.[28] The analog is 10 times less calcemic than 1α,25(OH)D3, when evaluated in vitamin D-deficient rats. It inhibited carcinogen-induced pre-cancerous lesions in mouse mammary gland organ culture. It also inhibited growth of ER+ MCF-7, ZR-75, and T47 D cells, and ER- UISO-BCA-4 human breast cancer cells in culture. The ability to induce

differentiation was evaluated in detail in ZR-75 and UISO-BCA-4 cells. The cells treated with 10^{-8}M 1α(OH)D_5 displayed altered cellular organization, resulting in the formation of ductlike structures in culture dishes. This phenomenon was accompanied by up-regulation of casein, ICAM, and nm23 expression.[29] Expression of these proteins is correlated with differentiation of breast cancer cells. Moreover, the differentiated cells (i.e., cells treated with D_5 analog) did not form adenocarcinoma in athymic mice as compared to the development of tumors in 100% of the control group. In addition, dietary supplementation of 1α(OH)D_5 inhibited the growth of breast cancer cells transplanted into mice.[29]

The mechanism of action of vitamin D is poorly understood.[8] In our studies with ER-positive and ER-negative breast cancer cells, 1α(OH)D_5 exerted relatively more cytostatic and cytotoxic effects on ER-positive cells. The reason for this differential effect is currently being pursued in our laboratory. At present, it is suggested that vitamin D induces its own receptors (i.e., vitamin D receptors [VDR]). We have shown that both 1,25$(OH)_2D_3$ and 1α(OH)D_5 induce VDR in breast carcinoma cell lines, as determined by immunocytochemistry, western blot analysis and RT-PCR.[29,30] Furthermore, the induction of VDR was accompanied by increased expression of TGFβ1 and TGFβ2. We hypothesize that 1α(OH)D_5, when administered to women with breast cancer or women with premalignant lesions, will induce differentiation of dedifferentiated and/or premalignant cells and thus will prevent progression and/or development of malignancy.

2.3 Pharmaceutical and chemical data

In order to synthesize 1α-hydroxyvitamin D_5, vitamin D_5 (24 ethyl vitamin D_5) was first synthesized from β-sitosterol. Vitamin D_5 was converted to 1α-hydroxyvitamin D_5 by following the Paaren-DeLuca hydroxylation sequence. The compound was crystallized and characterized by $_1$H NMR, mass spectroscopy, and UV and IR spectroscopy. The purity was checked by HPLC analysis. These results have previously been described in detail.[28] The structure of 1α-hydroxy-24, ethyl cholecalciferole (1α(OH)D_5) is shown in Appendix 3.

Preclinical studies, including cell culture experiments, pharmacokinetics, toxicity, and mechanism of action experiments, have been performed with crystalline compound; whereas, for the clinical trial, the crystalline compound will be prepared in gelatin capsules.

Dosage form preparation will be performed at the University of Illinois Hospital Pharmacy (Appendix 4 includes complete protocol for final dosage form preparation). To provide sufficient bulk to encapsulate the required microgram level dosage of study medications the crystalline 1α-hydroxyvitamin D_5 will be dissolved in alcohol and serial dilutions

performed to obtain appropriate concentrations. The 1α-hydroxyvitamin D_5 solution will then be absorbed into a suitable carrier (corn starch). After the alcohol is dried off and the material mixed, suitable quantities will be encapsulated. Study medications will be prepared on a weekly basis producing sufficient quantities of medication (42 capsules) to treat the six patients in the dosing cohort for one week. Additional capsules will be produced in every batch for content uniformity studies and periodic stability analysis.

3.0 STUDY HYPOTHESIS

We hypothesize that 1α(OH)D_5, when administered to women with breast cancer, will induce differentiation of dedifferentiated malignant cells and thereby prevent progression of malignancy. This is a phase I/II, single site, single arm, dose-escalation study.

4.0 OBJECTIVES

4.1 To determine the toxicity of 1α(OH)D5 in humans.

4.2 To obtain preliminary data on the efficacy of 1α(OH)D5 in advanced breast cancer patients.

5.0 DOSE AND ROUTE OF ADMINISTRATION

The present dosage schedule has been calculated based on our own preclinical data and recently reported Phase I trials with D_3 analogs for prostatic cancer.[31,32,33]

The major dose-limiting toxicity of vitamin D analogs has been hypercalcemia. Experimental evidence in animal studies indicates that the active metabolite was hypercalcemic at 2.99 nmole/kg body weight as compared to another analog in clinical trial EB1089 (seocalcitol), which was hypercalcemic at 5.5 nmole/kg body weight (BW). On the other hand, in preclinical toxicity studies we observed that 1α(OH)D_5 was non-calcemic at 11.65 nmole/kg BW. At 23.3 nmole/kg BW (10 μg/kg BW), there was an insignificant increase in calcium. As compared to 11.0 mg/dL in control rats, there was an 11.6 mg/dL in the 10-μg dose level.

Two Phase I/II clinical trials have been reported recently. In a calcitriol Phase I trial, 36 patients were given doses ranging from 2 to 10 μg every other day (QOD). At the highest dose, 3 out of 3 patients had hypercalcemia, whereas hypercalciurea was observed at all doses. No other toxicity was observed. The report concluded that calcitriol could be administered with tolerable toxicity.[31]

Another Phase II study was recently reported for advanced pancreatic cancer patients. In this study, 36 patients with advanced pancreatic cancer received once daily oral dose of seocalcitol (EB1089) with dose escalation every two weeks until hypercalcemia occurred. Once hypercalcemia occurred, the patients

were continued on maintenance therapy. The authors concluded that most patients tolerated 10-15 μg/day in a chronic treatment. Fourteen patients completed 8 weeks of treatment, whereas 22 patients were withdrawn due to clinical deterioration as a result of disease progression.[33]

Based on the experimental results indicating that 1α(OH)D_5 can be tolerated at a much higher concentration than other vitamin D analogs and the clinical studies described above, we expect no toxicity or hypercalcemia as a result of the proposed escalation protocol. Our protocol is designed with a starting dose of 5 μg/day (for a 70 kg person, this equals 0.035 μg/kg BW, with the highest dose of 35 μg/day/person (0.40 μg/kg BW). We do not anticipate that toxicity will be observed at these doses.

5.1 In the first group of six patients, a single, daily, oral dose of 1αhydroxyvitamin D_5 (5 μg/day) will be administered. Patients will be observed for signs and symptoms of hypercalcemia (see below for details). In the absence of any evidence of hypercalcemia, the 1α(OH)D_5 will be continued for 12 weeks (follow-up monthly blood tests will continue until week 28 with long-term follow-up testing to continue every two months for an additional six months or until death). If no toxicity is noted, the dose will be escalated in 5 μg increments, up to a total of 35 μg daily for 12 weeks (see Dose Escalation Schema below). In each of the seven dosing groups, six patients will be studied. There will be no dose escalation within the same cohort.

The following table shows the dose, route of administration, and escalation scheme. Between each dosing period listed below, there will be a minimum period of one week (7 days) during which results of the preceding dosing will be evaluated for safety parameters prior to initiating the next higher dosing level (see Section 5.3).

<u>Dose Escalation Schema</u>

Dose Level 1	Six(6) patients will be treated with oral administration of 5 μg/day for 12 weeks (84 days)
Dose Level 2	Six (6) patients will be treated with oral administration of 10 μg/day for 12 weeks (84 days)
Dose Level 3	Six (6) patients will be treated with oral administration of 15 μg/day for 12 weeks (84 days)
Dose Level 4	Six (6) patients will be treated with oral administration of 20 μg/day for 12 weeks (84 days)
Dose Level 5	Six (6) patients will be treated with oral administration of 25 μg/day for 12 weeks (84 days)

Dose Level 6 Six (6) patients will be treated with oral administration of 30 μg/day for 12 weeks (84 days)

Dose Level 7 Six (6) patients will be treated with oral administration of 35 μg/day for 12 weeks (84 days)

5.2 Duration of Treatment

5.2.1 Patients will be treated for a period of 12 weeks (follow-up blood tests will occur monthly until week 28 with additional follow-up tests every two months for an additional six months or until death).

5.2.2 It is estimated that the total number of evaluable patients will be entered within 24/36 months of the initiation of the study.

5.2.3 Patients demonstrating a progression of their disease as determined by the principal investigators will have their treatment discontinued and will be removed from the study. However, they will be followed for the 28 weeks of monthly blood tests (plus six months of blood tests at two month intervals or until death) for toxicity analysis.

5.2.4 An adequate trial requires three (3) months of treatment. An attempt will be made to keep patients on the study for the full three (3) months.

5.3 Dose Reduction and Stopping Criteria

5.3.1 Therapy may be discontinued at any time due to the development of any unacceptable toxicity.

If Grade 3 or higher non-hematologic or a Grade 4 hematologic toxicity develops, the study medication will be discontinued until recovery from toxicity. After complete recovery, the 1αhydroxyvitamin D_5 may be restarted at a dose of approximately one-half (minimum dose 5 μg/day) the original dose. Dosage reductions will be according to the following schedule:

Dose Cohort	**Reduced Dose**
5 μg/day	0 μg/day
10 μg/day	5 μg/day
15 μg/day	5 μg/day
20 μg/day	10 μg/day
25 μg/day	15 μg/day
30 μg/day	15 μg/day
35 μg/day	20 μg/day

5.3.2 If a Grade 3 or higher non-hematologic or a Grade 4 hematologic toxicity develops at the reduced dose level, the treatment will be discontinued and the patient will be removed from the study.

5.3.3 If two (2) out of six (6) patients develop Grade 3 non-hematologic or Grade 4 hematologic toxicity at the same dose level, no additional patient will be treated, and the study will be terminated.

6.0 PATIENT ELIGIBILITY

Forty-two (42) patients with metastatic breast cancer will be entered into this study. Patients with both estrogen receptor-positive and estrogen receptor-negative tumors will be eligible.

6.1 Inclusion criteria

6.1.1 Patients must have had histologically documented evidence of breast carcinoma.

6.1.2 Patients **must** have distant metastases (except brain metastases) and have a life expectancy of at least 3 months.

6.1.3 Patients must have failed at least one prior course of conventional treatment and must not be candidates for further treatment with anthracycline- or taxane-based therapy.

6.1.4 Patients must have signed an informed consent.

6.1.5 This study is confined to adult females age 18 or older.

6.1.6 ECOG Performance Status 0, 1, or 2 (see Appendix 1).

6.1.7 Patients must have no medical problems related to the malignancy that would pose an undue risk or that would limit full compliance with the study.

6.1.8 A minimum of 4 weeks must have elapsed since the completion of prior therapy, including hormonal therapy, chemotherapy, or radiation therapy, and patients must have fully recovered from such treatments.

6.1.9 Adequate baseline organ function as assessed by the following laboratory values within 30 days prior to study entry (with exception of corrected serum calcium and phosphorus, which are completed within seven days of study entry):

- Granulocyte count >1,500/mm^3, hematocrit >30%, and platelets >100,000/mm^3.

- Adequate renal function with estimated creatinine clearance >50 ml/min and or serum creatinine 2.5 or less.

- Corrected serum calcium level must be in the normal range (8.6-10.6 mg/dl) within seven days of study entry.
- No evidence of renal stones as determined by ultrasound of kidneys.
- Adequate liver function with SGOT, SGPT, LDH, and alkaline phosphatase <5x the upper limit of normal.
- PT and PTT not more than 1.5 times the upper limit of normal.
- bilirubin <2.0 mg/dl.

6.2 Exclusion criteria

6.2.1 Patients who are undergoing therapy with hormonal agents, cytotoxic agents, or any other therapy other than specified in this protocol. Concurrent focal radiation therapy with short-term supplemental steroids for spinal cord compression and/or severe bone pain unrelieved with standard pain medications is allowed.

6.2.2 Patients with brain metastases.

6.2.3 Patients with serious current illness, including untreated active infection.

6.2.4 Patients with any underlying conditions that would contraindicate therapy with study treatment (or allergies to D_5 used in this study).

6.2.5 Patients with prior or concomitant malignancies (except adequately treated basal cell carcinomas of the skin).

6.2.6 Patients with any other serious medical or psychiatric illness that would prevent informed consent.

6.2.7 Patients with breast cancer-related hypercalcemia (i.e., corrected serum calcium level outside the normal range of 8.6-10.6).

6.2.8 Patients with history of hypervitaminosis.

6.2.9 Patients who are either pregnant or lactating (all patients of childbearing potential will receive a pregnancy test within 7 days of study initiation).

6.3 Concomitant medication and treatment

All medications or treatments should be recorded. All questions regarding concomitant medications will be referred to the study investigators.

Medications and treatment not allowed

The following drugs and therapies are EXCLUDED while the patient is on study medication:

- Hormonal therapy, including steroids (However, patients are eligible to enroll if they are diabetic requiring insulin or if they are taking steroids as an adjunct to focal radiation therapy in cases of spinal cord compression and/or severe bone pain.)
- Chemotherapy
- Radiation therapy other than what is allowed (see 6.2.1)
- Megadose vitamin therapy
- Systemic therapy for hypercalcemia or biphosphinate treatment for any other therapy.

7.0 PATIENT EVALUATIONS

7.1 Pretreatment screening and baseline evaluations

A diagnosis of breast cancer must be confirmed by review of pathologic evaluation of prior biopsy and/or surgical specimen. The metastases must be documented by radiographic and/or nuclear medicine studies.

The following clinical and laboratory evaluations will occur within 30 days prior to study initiation (with the exceptions noted below). These screening evaluations must be reviewed prior to study treatment.

7.1.1 Complete history and physical examination. Include vital signs (blood pressure, pulse, temperature, and respiration), weight, and height.

7.1.2 Evaluation of Performance Status (PS) (see Appendix 1) and pain. Intensity of pain will be measured by verbal descriptors and visual analog scale (VAS) when Performance Status is measured.

7.1.3 Hematology: complete blood count (CBC) with differential, platelets, PT, and PTT.

7.1.4 Serum chemistries: glucose, electrolytes (Na+, K+, Cl-, CO_2), BUN, creatinine, total protein, albumin, bilirubin, alkaline phosphatase, LDH, SGOT, SGPT, magnesium, corrected serum calcium, phosphorus, cholesterol, and triglycerides.

7.1.5 Urinalysis.

7.1.6 Chest X-Ray (CXR).

7.1.7 Electrocardiogram (EKG).

7.1.8 CT scans of evaluable disease sites (within 60 days of study initiation).

7.1.9 Renal ultrasound (within 60 days of study initiation)

7.1.10 Bone scan (within 60 days of study initiation).

7.1.11 Pregnancy test of all patients of child bearing potential (within 7 days of study initiation). Patients will be instructed to use adequate birth control procedures throughout the study period.

7.2 Interval Evaluations

Patients will be followed in the clinic every week for the first four weeks and then every three weeks for the remainder of the study (total of 7 visits).

7.2.1 Interim history and targeted physical examination at each visit, which should include vital signs (blood pressure, pulse, temperature, and respiration), weight, and ECOG performance status (see Appendix 1).

7.2.2 Patients will be evaluated for bone pain during each visit. Intensity of pain will be measured by verbal descriptors and VAS at the same time as ECOG Performance Status is measured.

7.2.3 At each scheduled visit, each patient will be observed for possible adverse events, especially evidence of vitamin D toxicity (see Appendix 2). Any adverse event, whether observed by the investigative staff or reported by the patient, will be entered on the case report form and evaluated by the investigator as to severity and attribution. Adverse events will be documented by the criteria in Appendix 2.

7.2.4 Hematology: complete blood count (CBC), differential, and platelets at every study visit.

7.2.5 Serum chemistries: glucose, electrolytes (Na+, K+, Cl-, and CO2), BUN, creatinine, total protein, albumin, bilirubin, alkaline phosphatase, LDH, SGOT, SGPT, calcium, phosphorus, and serum lipids will be checked at every study visit. Also, 1α(OH)D5 levels and other indicators of increased blood calcium will be determined.

7.2.6 Appropriate radiographic and nuclear imaging studies will be performed at week 12 and week 28, or sooner if disease progression is suspected (see Section 11.0 and Section 17.0).

7.3 Post-treatment evaluations

7.3.1 The same evaluations as section 7.2 will be performed at one (1) month intervals for an additional four months (until week 28)

7.3.2 The same evaluations as section 7.2 will be performed every two months for an additional period of six (6) additional months.

7.3.3 Radiographic and nuclear medicine studies will be repeated at week 28 and at the end of the sixth (6) months, or sooner if disease progression is suspected.

7.4 Risks to subjects

7.4.1 Risks to subjects and measures to minimize them are listed in the table below.

Procedure	**Risks**	**Measures to Minimize Risks**
Intake of $1\alpha(OH)D_5$	Hypercalcemia, which may cause fatigue, upset stomach, constipation, nausea, bone pain, increased urination, increased thirst, weight loss, appetite loss, vomiting, low pulse rate, itching, muscle weakness, slow reflexes. At very high levels, subjects may experience confusion, mental illness, seizure or coma. Hyperphosphatemia resulting in hypocalcemia with symptoms of muscle weakness. If left untreated, could result in kidney failure	Subjects are observed for evidence of vitamin D toxicity at each scheduled visit. Blood tests will be performed every week for the first four weeks and then every three weeks during the course of treatment. At the onset of any symptoms, the subject will be tested for blood calcium and treated based on its level. Treatment usually consists of the following: 1. Administration of intravenous fluids to restore hydration. 2. Maintaining the subject's mobility and activity level consistent with the subject's presenting symptoms. 3. Administration of drugs such as diuretics or biphosphonates to lower serum calcium level.

Procedure	Risks	Measures to Minimize Risks
Venipuncture	Pain and bruising at puncture site; rarely fainting	Application of pressure at puncture site and elevation of extremity following venipuncture. Subject will be seated or lying down during blood draw.
Maintenance of data linked to an identifiable individual	Breach of confidentiality	Study participants will not be identified by name on any study documents. They will be identified by initials and a patient identification number. Investigators will keep a separate log of patients' codes, names and addresses.

8.0 TREATMENT MODIFICATION AND DISCONTINUATION ACCORDING TO LEVEL OF TOXICITY

The current NCI Common Terminology Criteria for Adverse Events table (Appendix 2, available online at http://ctep.cancer.gov/reporting/ctc.html), also applicable to vitamin D-induced hypercalcemia, will be used to grade the severity of adverse experience and to achieve consistency in response to drug/treatment toxicities. Toxicity will be graded on a 1-4 grading scale. If a toxicity is experienced, the treatment level or dose will be modified (if applicable) as outlined below according the grade toxicity observed.

8.1 Treatment modification and general management of toxicities

8.1.1 For any Grade 1 toxicity, there will be no dose modification.

8.1.2 If a Grade 2 toxicity develops, the investigator should continue the treatment with careful monitoring.

8.1.3 In general, if a Grade 3 non-hematologic or Grade 4 hematologic toxicity is obtained, treatment will be withheld until the Grade reaches 1 or less. Repeat tests to confirm values within 72 hours will be required.

8.1.4 After recovering from Grade 3 non-hematologic or Grade 4 hematologic toxicity, test medication will be restarted at a dose approximately one-half of the dose that caused the toxicity. If the original toxicity reoccurs at the lower dose level, treatment will be discontinued and the patient will be removed from study.

8.1.5 The development of Grade 3 non-hematologic or Grade 4 hematologic toxicity in two (2) out of six (6) patients at any given dose level will result in the termination of all dosing at that level and no additional higher dose cohorts will be entered into the study.

This dose level is defined as a dose limiting toxicity with the maximum tolerated dose (MTD) being one dose level below (see Section 11.1 for description of MTD).

8.2 Symptomatic therapy for toxicity

Along with reduction of the dose level, any required symptomatic therapy for hypercalcemia or other toxicity may be administered if deemed necessary by the investigators. All medications or other treatments administered will be recorded in the appropriate Case Report Form section.

9.0 CRITERIA FOR DISCONTINUATION OF STUDY PATIENTS

9.1 Criteria for treatment discontinuation

The investigator will encourage study subjects to remain in the study through completion. However, should the subject decide to withdraw, all efforts will be made to complete and report the observations as thoroughly as possible, including a complete final evaluation at the time of the subject's withdrawal with an explanation of why the subject is withdrawing from the study.

Participation in this study can be discontinued for any of the following reasons listed below:

9.1.1 Progressive disease despite an adequate trial with study medication.

9.1.2 A major, unexpected, or life-threatening event.

9.1.3 Generalized impairment or mental incompetence which would render the patient unable to understand his/her participation in the study.

9.1.4 If, in the investigator's medical judgement, further participation would be injurious to the subject's health or well-being.

9.1.5 Patient request or noncompliance.

An explanation will be recorded for any patient who has been taken off treatment, and the appropriate section of the Case Report Form will be completed.

9.2 Criteria for study modification and/or discontinuation

9.2.1 Study Modification

If preliminary or interim analysis indicates that modifications should be made in the experimental design, dosages, patient selection, etc., these changes will be made in the form of an amendment after

consultation with the sponsoring agency (Department of Defense [DOD]) and the UIC Cancer Center statistician. Changes listed in the Amendment will not be initiated until approved by the Institutional Review Committees.

9.2.2 Study Discontinuation

If the principal investigators should discover conditions arising during the study which indicate the study be terminated, an appropriate schedule for termination will be instituted and appropriate authorities notified.

10.0 ADVERSE EXPERIENCES

Both serious adverse events and serious and unexpected adverse events will be reported to the Institutional Review Board (IRB) and the Food and Drug Administration (FDA) in accordance with existing Federal Regulations. All serious adverse events and serious and unexpected adverse events will be reported immediately to:

Manley A. Paulos, Ph.D.
Vice President, Operations
Komodo Clinical Trials Management, Inc.
520 Brookview Ct. Suite 202
Auburn Hills, Michigan 48326
Voice: (248) 335-6650
Fax: (248) 335-6296
Manleypaulos@komodo-inc.com

Komodo staff will verify classification of the event, code events, and provide all written and fax/telephone notifications of events to regulatory authorities.

The event will also be immediately reported by telephone to the USAMRMC Deputy Chief of Staff for Regulatory Compliance and Quality (301-619-2165) (non-duty hours call 301-619-2165 **and** send information by facsimile to 301-619-7803). A written report will follow the initial telephone call within 3 working days. Address the written report to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

11.0 EVALUATION OF RESPONSE/ENDPOINTS

11.1 Toxicity

The criteria for grading toxicity (Grade 1 through Grade 4) are found in the Toxicity Tables (see Appendix 2).

The development of a Grade 3 non-hematologic or Grade 4 hematologic toxicity in two (2) out of six (6) patients at any given dose level is defined

as a dose limiting toxicity (DLT). The maximum tolerated dose (MTD) is defined as that dosage level immediately below the level at which the DLT was observed.

11.2 Clinical response

11.2.1 Evaluation criteria

Bone Scans

Care is needed in the interpretation of a bone scan report of increased radionuclide uptake if the patient is clinically stable or improving (flare phenomenon).

Tumor Measurements

All tumor measurements must be recorded in centimeters and should consist of the longest diameter and the perpendicular diameter at the widest portion of the tumor.

Patients will be considered evaluable for toxicity if they receive one or more doses of study medication.

11.2.2 Criteria for response and definitions

Complete Response (CR) (all of the following):

- Complete disappearance of all tumor masses. Osteolytic bone lesions must demonstrate recalcification.
- Normalization of all laboratory parameters related to the patient's disease or to toxicity of the therapy.
- No new lesions may appear.
- Resolution of all symptoms related to cancer.

Partial Response (PR) (any of the following criteria):

- A >50% decrease in the sum of the products of the diameters of any measurable lesions.
- Recalcification of ≥1 osteolytic lesion.
- A reduction by >50% in the number of areas of increased uptake on bone scan.

and ALL of the following:

- No simultaneous increase in the size of any evaluable lesion or appearance of any new lesions.

- No deterioration in weight (10%), symptoms, or performance status (more than one score level), which is not explained by drug toxicity.

- Response duration will be measured from the time of initial documentation of response.

Stable Disease (SD) (all of the following):

- There may be no appearance of new lesions. No measurable lesion may enlarge by ≥25%.

- No elevation of serum tumor markers to >50% over baseline.

- Osteolytic lesions, if present, must not worsen.

- Osteoblastic lesions, if present, must remain stable on bone scan.

- The patient must have no significant deterioration in performance status (greater than 1 score level), weight (>10%), or symptoms.

Progressive Disease (PD) (any of the following):

- Unequivocal increase of >25% in size of any measured lesion.

- Appearance of new malignant lesions.

- Significant deterioration in weight (>10%), performance status (>1 score level), or symptoms.

Recurrence/Relapse:

- The reappearance of old lesions in patients who have achieved complete response, or, for patients with partial response, an increase of 25% or more in the sum of the products of the diameters of all measured lesions.

11.2.3 Response duration will be measured from the time of initial documentation of response.

12.0 DOCUMENTATION, RECORD KEEPING, CASE REPORT FORMS

The investigator will maintain adequate records so that the conduct of the study can be fully documented and monitored.

Copies of protocols, case report forms (CRFs), patient medical records, test result originals, and all documents relevant to the conduct of the study will be kept on file by the investigator for five years after all investigational use of

product is discontinued and the FDA is so notified or until five years after a Product Licensing Application (PLA/ELA) is approved. Study documents will not be destroyed. For FDA and sponsor inspections, it will be necessary to have access to complete study patient records, provided that patient confidentiality is maintained.

The investigator will obtain a separate release of medical information form to be signed by the study patient in order to facilitate access to the patient's medical records should the patient be hospitalized at an institution with which the study investigator is not associated.

A record will be kept of all patients who have been screened for the study and subsequently deemed ineligible. The reason for ineligibility must be recorded.

13.0 DATA COLLECTION AND STUDY MONITORING

13.1 Data collection

Case report forms (CRFs) will be used for each patient entered into the study. Study participants will NOT be identified by name on any study documents. Patients will be identified by a patient identification number (PIN). Investigators will keep a patient code list accessible.

14.0 BIOSTATISTICAL CONSIDERATIONS

The end-points of the phase I/II study are response rate and determination of the maximum tolerated dose (MTD), based on the occurrence of Grade 3 non-hematologic or Grade 4 hematologic toxicity in 2 of 6 patients at any given dose level. Should the MTD be reached before the accrual of 42 patients, the study will continue to accrue patients at one dose below the dose at which 2 of 6 patients experienced Grade 3 non-hematologic or Grade 4 hematologic toxicity.

Since the endpoints of this study are toxicity and finding both the MTD and an appropriate dose for 1-αhydroxyvitamin D_5, any withdrawal of a patient from any of the group will require that another patient be provided to replace that patient.

15.0 ETHICAL CONSIDERATIONS

The investigator will ensure that the study is conducted in full conformance with the FDA and the U.S. Department of Health and Human Services Office for Human Research Protections (OHRP) standards for human research.

15.1 Informed consent

All study participants must sign an informed consent form. The investigators will inform all subjects as to the nature, aims, duration, potential hazards, and procedures to be performed during the study and that his or her medical records may be reviewed by the independent monitor, UIC IRB, DOD, and/or the FDA. The investigators must also explain that the patients are completely free to refuse to enter the study or

to withdraw from it at any time. The protocol will be discussed in detail with all potentially eligible patients. All revisions of the protocol must be reflected in the consent form and reviewed by the IRB.

A translator is on staff and available to translate consent forms. This person is fluent in Spanish (the most widely used second language) and several other languages. For lesser used languages (Farsi, Cantonese, etc.), the university keeps a list of staff members fluent in these languages, and a translation can be arranged into most languages.

15.2 Patient confidentiality

All reports and patient samples will be identified only by a coded number to maintain patient confidentiality. All records will be kept confidential to the extent permitted by law. The investigators will keep a separate log of patients' codes, names, and addresses. Documents identifying the patient by name (informed consent) will be kept in strict confidence.

16.0 CRITERIA AND PROCEDURES, FOR PROTOCOL MODIFICATION AND STUDY TERMINATION

Modifications that may affect the safety of the study patient, or that may alter the scope of the investigation, the scientific quality of the study, the study design, dosages, duration of therapy, patient assessments (added evaluation that poses potential risk or inconvenience to the patient), number of patients, and patient eligibility criteria, may be made only after appropriate consultation with the UIC IRB and DOD. If the consensus is to revise the current protocol, a formal List of Changes will accompany the amended protocol, and these will be submitted to the IRB, the DOD, and any other committee as indicated. The changes to the protocol will be submitted to the FDA only in the Annual Report, which is provided within 60 days of the IND filing anniversary.

The investigators reserve the right to terminate the study at any time. If this becomes necessary, appropriate procedures for continuing the long-term follow-up requested by the regulatory agencies will be arranged after review and approval by both parties.

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17.0 SCHEDULE OF TESTS AND PROCEDURES*

Tests and procedures	Pre-study	End Wk 1	End Wk 2	End Wk 3	End Wk 4	End Wk 7	End Wk 10	End Wk 12	End Wk 16	End Wk 20	End Wk 24	End Wk 28	Q 2 months for six months or until death
History	X												
Physical Exam	X	X	X	X	X	X	X	X	X	X	X	X	X
Weight	X	X	X	X	X	X	X	X	X	X	X	X	X
Height	X												
Performance status	X	X	X	X	X	X	X	X	X	X	X	X	X
Evaluation of pain or other symptoms	X	X	X	X	X	X	X	X	X	X	X	X	X
Evaluation of toxicity		X	X	X	X	X	X	X	X	X	X	X	X
CBC, diff, PLT	X	X	X	X	X	X	X	X	X	X	X	X	X
Chemistry panel	X	X	X	X	X	X	X	X	X	X	X	X	X
Corrected Serum calcium[1]	X	X	X	X	X	X	X	X	X	X	X	X	X
Serum phosphate[2]	X	X	X	X	X	X	X	X	X	X	X	X	X
Urinalysis	X												
Chest X-Ray	X												
EKG	X												
Bone scan	X							X				X	**
Ultrasound of kidney	X							X				X	**
CT scan	X							X				X	**
Pregnancy test[3]	X												
D5 levels		X	X	X	X	X	X	X	X	X	X	X	

* Weekly tests will be run for 28 weeks, though treatment will only last 12 weeks (plus six months of follow-up tests every two months). All tests will be run at the end of the week indicated plus or minus two (2) days.
** Radiographic and nuclear medicine studies as indicated above or sooner if disease progression is suspected.
[1, 2, 3] will be done within one week of initiation of the study.

APPENDIX 1

ECOG CRITERIA FOR ESTIMATION OF PERFORMANCE STATUS

CRITERIA FOR ESTIMATION OF PERFORMANCE STATUS

Grade Scale

0 Fully Active, able to carry on all pre-disease performance without restriction.

1 Ambulatory, capable of light or sedentary work. Restricted in physically strenuous activity.

2 Ambulatory, capable of all self-care, but not of work activities; up and about more than 50% of waking hours.

3 Capable of only limited self-care; confined to bed or chair more than 50% of waking hours.

4 Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.

APPENDIX 2

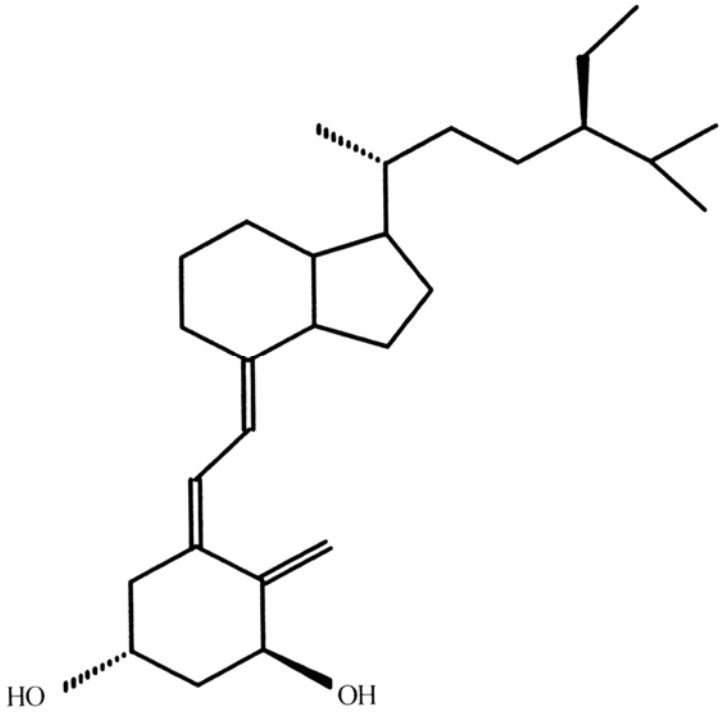
TOXICITY TABLE

THE NCI COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS (CTCAE)

Available online at http://ctep.cancer.gov/reporting/ctc.html

APPENDIX 3

STRUCTURE OF 1α-HYDROXY-24, ETHYL CHOLECALCIFEROLE (1α(OH)D_5)



Chemical structure and high-pressure liquid chromatography (HPLC) profile of 1αhydroxyvitamin D_5 [1α(OH)D_5]. The agent was dissolved in acetonitrile (200 μg/mL), and l0-μL aliquots were injected on a Suplex PKB-1OO HPLC column. The retention time for 1α(OH)D_5 was about 34 minutes.

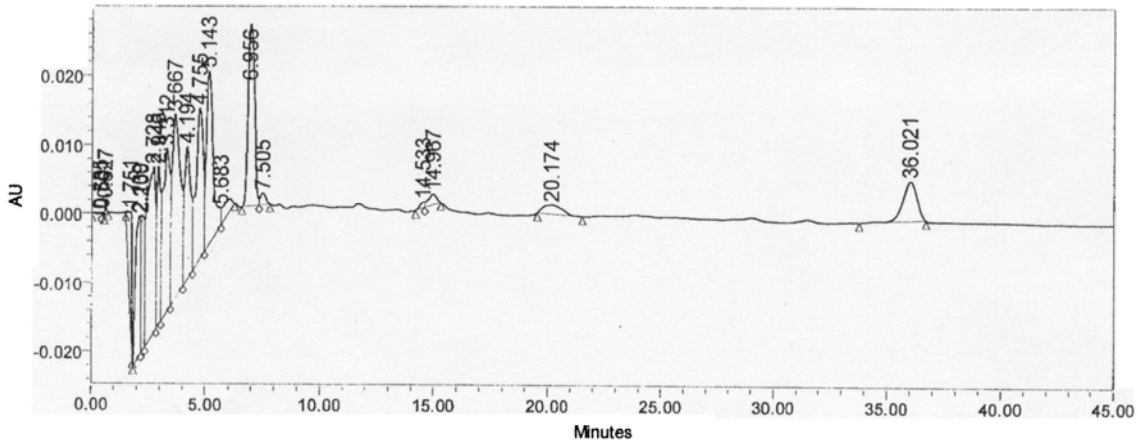
Sample Report

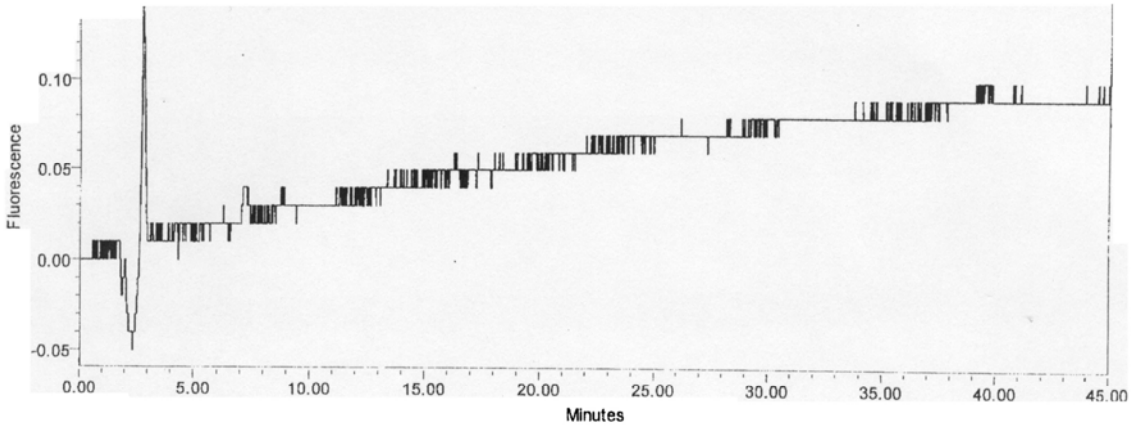
Reported by User: System

Project Name: Vit_D1

SAMPLE INFORMATION

Sample Name:	D5 std	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	2/13/03 10:50:41 AM
Vial:	12	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	2/21/03 1:00:06 PM
Injection Volume:	100.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	474 Ch1, 486
Sample Set Name:	D5 day7	Proc. Chnl. Descr.:	





	Peak Name	RT	Area	% Area	Height
1		0.385	5321	0.10	348

Report Method: Result Set Report Printed 1:00:09 PM 2/21/03

001--082

Page: 1 of 6

Sample Report

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
2		0.501	2246	0.04	489
3		0.627	8595	0.17	1883
4		1.751	168922	3.26	16641
5		2.139	321667	6.21	20220
6		2.200	192888	3.72	19810
7		2.728	638507	12.32	24279
8		2.946	268769	5.19	23157
9		3.312	515961	9.96	23900
10		3.667	687136	13.26	26898
11		4.194	377511	7.29	19414
12		4.755	482298	9.31	21973
13		5.143	514082	9.92	25195
14		5.683	58130	1.12	2415
15		6.956	524665	10.13	26296
16		7.505	30655	0.59	1734
17		14.533	13187	0.25	860
18		14.967	42976	0.83	1613
19	MDA	18.000			
20	MDA	18.000			
21		20.174	75701	1.46	1306
22		36.021	252242	4.87	5786

Report Method: Result Set Report Printed 1:00:09 PM 2/21/03 001--083 Page: 2 of 6

APPENDIX 4

Protocol for Compounding Final Dosage Form

PROCEDURE FOR VITAMIN D5 STORAGE AND PREPARATION OF CAPSULES TO BE USED IN CLINIC UNDER IND # 56,509

1. Pure Vitamin D5 (drug substance) is stored at -75 °C (Revco freezer model #ULT 390-5-A14 Revco Elite) with continuous graphic recording of temperature. Graphic recording charts are changed regularly and maintained on file.

2. On the day capsules are to be prepared, Vitamin D5 is removed from the freezer for formulation in cornstarch. D5 is dissolved in ethanol for dilution in cornstarch, according to the procedure outlined by Dr. Raju Mehta.

    a. Weigh 1 mg Vitamin D5 (using Acculab V-1 Electronic Balance)
    b. Dissolve D5 in 1 mL 100% ethanol, USP
    c. Add 1 mL of corn oil, USP
    d. Add 0.5 mL Tenox® for a final concentration of 1 mg/2.5 mL

3. Preparation of 5 μg dosage:

    a. Weigh 3 gm of cornstarch, NF
    b. Using a syringe, measure 0.25 mL D5 solution (0.1 mg)
    c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 5 μg D5/150 mg cornstarch
    d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

4. Preparation of 10 μg dosage:

    a. Weigh 3 gm of cornstarch, NF
    b. Using a syringe, measure 0.5 mL D5 solution (0.2 mg)
    c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 10 μg D5/150 mg cornstarch
    d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

5. Preparation of 15 μg dosage:

    a. Weigh 3 gm of cornstarch, NF
    b. Using a syringe, measure 0.75 mL D5 solution (0.3 mg)
    c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 15 μg D5/150 mg cornstarch
    d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

6. Preparation of 20 μg dosage:

    a. Weigh 3 gm of cornstarch, NF
    b. Using a syringe, measure 1.0 mL D5 solution (0.4 mg)
    c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 20 μg D5/150 mg cornstarch
    d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

7. Preparation of 25 μg dosage:

    a. Weigh 3 gm of cornstarch, NF
    b. Using a syringe, measure 1.25 mL D5 solution (0.5 mg)
    c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 25 μg D5/150 mg cornstarch
    d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

8. Preparation of 30 μg dosage:

    a. Weigh 3 gm of cornstarch, NF
    b. Using a syringe, measure 1.5 mL D5 solution (0.6 mg)
    c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 30 μg D5/150 mg cornstarch
    d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

9. Preparation of 35 μg dosage:

    a. Weigh 3 gm of cornstarch, NF
    b. Using a syringe, measure 1.75 mL D5 solution (0.7 mg)
    c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 35 μg D5/150 mg cornstarch
    d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

Prepared capsules will be immediately placed in vials (7 per vial), labeled with the CTM label, and transported to the clinical sites. Ten (1 a) capsules per batch will be placed in a vial with the CTM label, and marked “RETAINS”, and stored at room temperature under lock and key in the pharmacy.

Appendix 3: Publications

PERGAMON

European Journal of Cancer 36 (2000) 780–786

European Journal of Cancer

www.ejconline.com

Induction of differentiation by 1α-hydroxyvitamin D_5 in T47D human breast cancer cells and its interaction with vitamin D receptors

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Abstract

The role of the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), in cell differentiation is well established. However, its use as a differentiating agent in a clinical setting is precluded due to its hypercalcaemic activity. Recently, we synthesised a relatively non-calcaemic analogue of vitamin D_5, 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), which inhibited the development of carcinogen-induced mammary lesions in culture and suppressed the incidence of chemically induced mammary carcinogmas in rats. In the present study, we determined the differentiating effects of 1α-$(OH)D_5$ in T47D human breast cancer cells and compared its effects with $1,25(OH)_2D_3$. Cells incubated with either 10 or 100 nM of the analogues inhibited cell proliferation in a dose-dependent manner, as measured by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. Similar growth-inhibitory effects were also observed for $MCF10_{neo}$ cells. Both vitamin D analogues induced cell differentiation, as determined by induction of casein expression and lipid production. However, $MCF10_{neo}$ cells failed to respond to either vitamin D analogue and did not undergo cell differentiation. Since the cell differentiating effect of vitamin D is considered to be mediated via the vitamin D receptor (VDR), we examined the induction of VDR using reverse transcriptase–polymerase chain reaction (RT-PCR) in both cells. The results showed that, in T47D cells, both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ induced VDR in a dose-dependent manner. Moreover, both analogues of vitamin D upregulated the expression of vitamin D response element-chloramphenicol acetyl transferase (VDRE-CAT). These results collectively indicate that 1α-$(OH)D_5$ may mediate its cell-differentiating action via VDR in a manner similar to that of $1,25(OH)_2D_3$.

Keywords: Vitamin D; Breast cancer cells; Differentiation; T47D; MCF10

1. Introduction

The research on vitamin D_3 and related compounds is currently at its apex. A vast amount of evidence has been collected, implicating the essential involvement of vitamin D metabolites in several cellular processes. The active metabolite 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) and related compounds suppress the development and progression of breast cancer and other carcinomas *in vivo* [1,2], inhibit the metastatic spread of tumour cells [3–5], and promote differentiation of breast cancer cells [6–8]. However, the calcaemic side-effects of $1,25(OH)_2D_3$ have prevented its application as a pharmaceutical agent. In recent years, considerable attention has been given to the development of vitamin D_3 analogues capable of inducing cell differentiation without systemic hypercalcaemia [8–10]. Many structural modifications are known to enhance several-fold the differentiating potency of vitamin D_3 analogues in normal (usually keratinocyte) or malignant (usually leukaemia) cell lines. Little attempt, however, has been made to evaluate vitamin D analogues of other series such as vitamin D_2, D_4, D_5 and D_6. This structural classification is based on the differences encountered in the side chain. Earlier studies reported that vitamin D_5 was the least toxic of vitamins D_2 through to D_6 [11].

During the past 2 years, we have been studying the role of 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), an analogue of vitamin D_5 (24-ethyl-vitamin D_3), on breast cancer cell differentiation. We have characterised its calcaemic

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activity in vitamin D-deficient Sprague–Dawley rats [12]. The analogue $1\alpha(OH)D_5$ was synthesised from sitosterol acetate and was found to be less calcaemic than vitamin D_3. It was observed that $1\alpha(OH)D_5$ was effective against the development of carcinogen-induced mammary lesions in mouse mammary gland organ cultures [12]. In a more recent study, we observed that $1\alpha(OH)D_5$ inhibited incidence and tumour multiplicity of N-methyl-N-nitrosourea-induced mammary adenocarcinoma in rats (data not shown). These results clearly demonstrate that this vitamin D analogue might be a good candidate in the prevention of mammary carcinogenesis. In the present study, we evaluated the effects of $1\alpha(OH)D_5$ on cell differentiation and proliferation in oestrogen receptor (ER)-positive T47D breast cancer cells and compared the effects of the D_5 analogue with the active metabolite of vitamin D_3, $1,25(OH)_2D_3$. Moreover, we compared the effects of vitamin D analogues between ER+ T47D cells and ER- $MCF10_{neo}$ cells. Both cell lines are negative for functional p53 [13].

It is well known that the nuclear activity of vitamin D_3 is based on the interaction of the vitamin D active metabolite, $1,25(OH)_2D_3$, with the vitamin D receptor (VDR) [14]. The VDR is a nuclear receptor that belongs to the superfamily of ligand-dependent transcription factors and is expressed in all the vitamin D target tissues. VDR mediates its action by conjugating with the Retinoid X Receptor (RXR) [15–17]. The VDR-RXR dimer, once formed, is capable of recognising the vitamin D response element (VDRE) in the promoter region of the gene. The VDRE is composed of direct repeats of 6 DNA bases separated by 3-base intervening sequences [18]. Vitamin D appears to play an important role in stabilising and transactivating the VDR/RXR–VDRE complex [19,20]. Its interaction with VDR, therefore, represents the central step in the transmission of a signal to the transcription machinery, resulting in activation or suppression of transcription of genes leading ultimately to differentiation. We recently showed that the normal human breast epithelial cells lacking functional VDR do not respond to vitamin D to induce cell differentiation. However, transient transfection of VDR in these HBL-100 cells resulted in increased association of VDR-VDRE, as measured by the CAT reporter assay [21]. In the present study, we compared the effects of $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ on the transactivation of VDR–VDRE in T47D cells.

2. Materials and methods

2.1. Cells

The breast epithelial cell line, $MCF10_{neo}$, and human breast cancer cell line, T47D, were obtained from the American Type Culture Collection (Rockville, MD, USA). The $MCF10_{neo}$ cells were maintained in minimum essential medium with Earl's salts (MEME) medium supplemented with 10% fetal bovine serum (FBS), whereas T47D cells were maintained in RPMI supplemented with 0.2 I.U. bovine insulin/ml and 10% FBS. The monkey renal cancer CV-1 cells were maintained in MEME with 10% FBS supplement.

2.2. MTT assay

The cells were seeded in a 96-well/plate at a density of 500 cells/well in 100 μl/well of cell culture medium supplemented with 10% steroid-stripped serum. 24 h after seeding, the cells were incubated with 10 and 100 nM concentrations of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, respectively. The medium was changed every 3 days. After 7 days, the cultures were used for the dimethylthiazolyl-2,5-diphenyltetrazolium Bromide (MTT) assay. MTT (5 mg/ml in phosphate buffered serum (PBS)) was added to the wells (15 μl/well) and incubated at 37°C for 2 h. The stop solution (20% sodium dodecyl sulphate (SDS) in 50% N,N-dimethylformamide) was then added (100 μl/well) and incubated for an additional 2 h. The plates were scanned at 590 nm OD, and the results for each treatment group were averaged.

2.3. Immunohistochemistry

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS and fixed in 10% formalin for 5 min. The fixed cells were further incubated in cold methanol for 3 min and acetone for 2 min. After blocking the cells with a protein block/normal goat serum (BioGenex, San Ramos, CA, USA), they were incubated with casein antibody (100 μg/ml) (Accurate Chemical and Scientific Corp. Westbury, NY, USA) for 2 h. The cells were then incubated with secondary anti-mouse biotinylated antibody for 30 min, followed by streptavidin–peroxidase complex and 3,3′-diaminobenzidine (DAB) solution as chromogen. Appropriate controls were performed to rule out non-specific staining with secondary antibody.

2.4. Lipid assay

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS, and fixed by incubating in cold methanol for 3 min and propylene glycol for 2 min. The cells were, at this point, stained with Oil Red O' for 30 min and rinsed in isopropyl alcohol then de-ionised water. Haematoxylene staining for 30 s and Scott solution rinse completed the assay.

2.5. RNA isolation and RT-PCR

The cells were incubated with 1, 10 or 100 nM $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 3 days. The medium

from the tissue culture flasks was removed and the cells were treated with RNA-Zol B (Tel-Test Inc., Friendswood, TX, USA). RNA were isolated according to the manufacturer's instruction and quantified spectrophotometrically. Reverse transcription (RT) and PCR were carried out using Advantage RT for PCR and Advantage cDNA PCR kit (Clontech Inc., Palo Alto, CA, USA). Primer sequences for VDR were selected and custom-synthesised by Oligos Etc. The sense primer was 5′-GGA GTT GCT GTT TGT TTG AC, and the antisense primer was 5′-CTT CTG TGA GGC TGT TTT TG. The primer for the housekeeping gene *G3PDH* was purchased from ClonTech. The touchdown PCR procedure was employed with minor modifications [22]. The first strand cDNA was heated at 94°C for 1 min followed by denaturation at 94°C for 45 sec, annealing at 68–66–64–62–60°C for 45 sec each time and extension at 72°C for 2 min, for 26 cycles. The final cycle was followed by a 7-min extension step at 72°C to ensure that the amplified DNA was double stranded. The absence of contaminant was routinely checked by RT-PCR assays of negative control samples (sterile buffer, provided in the kit). The PCR products were separated on 1.5% agarose gel at 64 volts for 3 h, stained with ethidium bromide and visualised by ultraviolet (UV)-transillumination.

2.6. Transient transfection

The reporter construct VDRE-tk-CAT was prepared by inserting a copy of VDRE into the *Bam*HI site of the pBLCAT$_2$ as previously described [23]. For transfection, 1×10^5 CV-1 cells were plated in 24-well plates. Transfections were carried out using the calcium phosphate precipitation procedure. Briefly, 100 ng of VDRE-tk-CAT reporter plasmid, 250 ng of β-galactosidase (β-gal) expression vector, and 500 ng of VDR expression vectors were mixed with carrier DNA (pBluescript) to 1 μg of total DNA per well. The CAT activity was normalised for transfection efficiency by the corresponding β-gal activity.

3. Results

3.1. Effect of 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ on cell proliferation

The breast epithelial cells MCF10$_{neo}$ and breast cancer cells T47D were incubated with the vitamin D analogues for 7 days in culture. After this, the effects of vitamin D analogues were evaluated by the MTT assay. The results indicated a 31% and 50% growth inhibition for MCF10$_{neo}$ at 10 and 100 nM of 1,25(OH)$_2$D$_3$ concentrations, respectively, as compared with 50% and 72% inhibition with 1α(OH)D$_5$ at 10 and 100 nM, respectively (Fig. 1a). The ER-positive, T47D cells showed a 29% and 52.5% growth inhibition after being exposed for 7 days to 1,25(OH)$_2$D$_3$ at 10 and 100 nM, respectively. Unlike MCF10$_{neo}$ cells, T47D cells did not exhibit increased growth suppression when exposed to 1α(OH)D$_5$. Both analogues suppressed growth of T47D cells by approximately 30% and 50% at low and high concentrations, respectively (Fig. 1b). These results suggest that both 1α(OH)D$_5$ and 1,25(OH)$_2$D$_3$ are comparable in producing antiproliferative effects in breast cancer cells.

3.2. Induction of differentiation of breast cancer cell lines

Since one of the major recognised functions of vitamin D is induction of cell differentiation, we evaluated the effects of both analogues on the induction of differentiation in both cell lines. As markers of cell differentiation, we used casein and lipid. Casein expression was measured by immunocytochemistry using casein antibodies. Results showed that, for T47D cells, casein was expressed in less than 10% of the control cells.

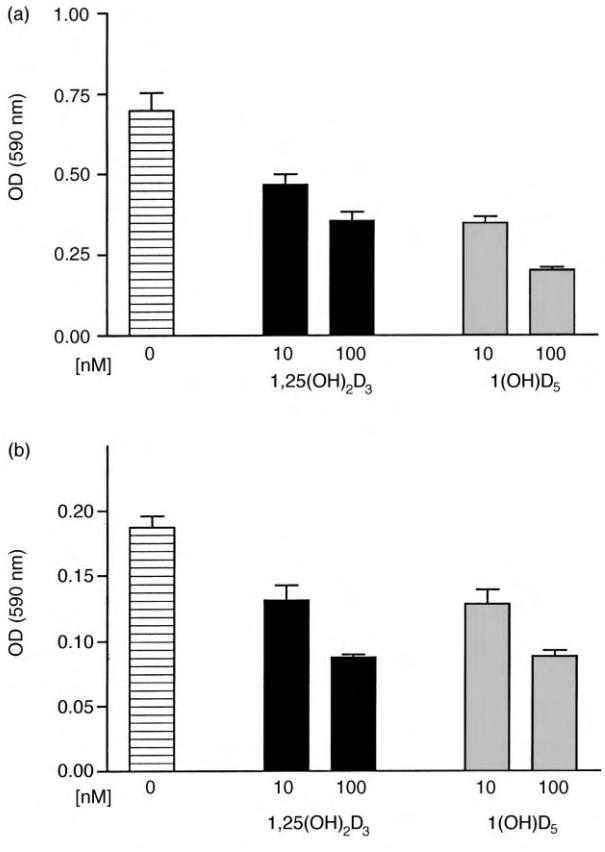

Fig. 1. Effects of 1,25(OH)$_2$D$_3$ and 1α (OH)D$_5$ on the proliferation of MCF10$_{neo}$ cells and T47D cells. The MTT assay was carried out using duplicate cultures and the experiments were repeated three times. The error bars represent the standard deviation. (a) MCF10$_{neo}$ cells; (b) T47D cells.

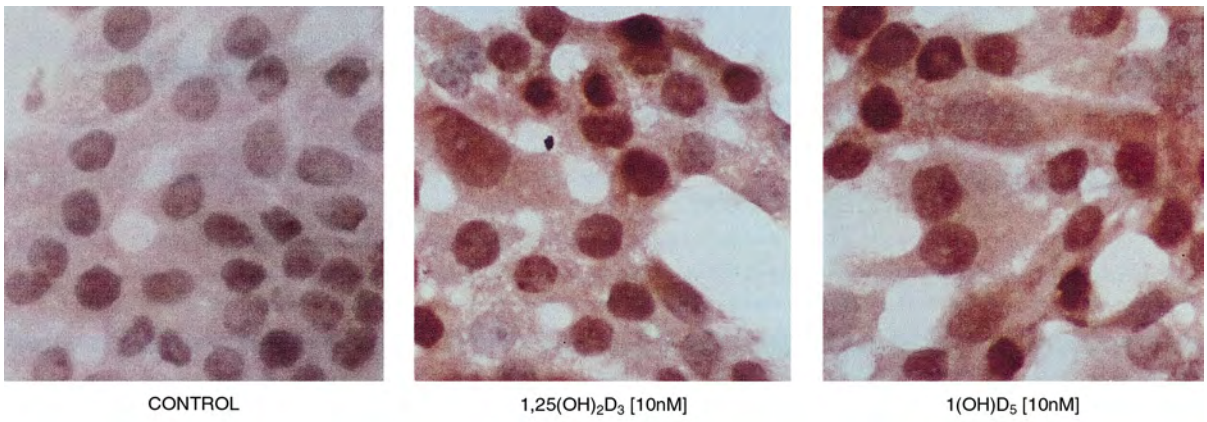

Fig. 2. Effect of vitamin D analogues on casein expression in T47D cells. Immunohistochemical staining for casein expression was carried out as previously described in the presence or absence of the vitamin D analogues.

After 7 days treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, the intensity and number of cells expressing casein increased to approximately 70 and 85% at 10 and 100 nM concentrations, respectively. No difference was noticed between the effects of D_3 or D_5 analogues (Fig. 2 and data not shown). Similarly, there was a dramatic increase in the expression of lipid production in T47D cells after 7 days of treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ (Fig. 3). These results indicated that both vitamin D analogues induce cell differentiation in T47D cells. In contrast, the $MCF10_{neo}$ cells, tested for the same markers of differentiation, did not show any presence or induction of either casein or lipids in the control cells or in cells exposed to vitamin D_3 or D_5 (data not shown).

3.3. Transactivation of VDRE

The VDRE transactivation activity of the vitamin D analogues was determined using the *CAT* reporter gene containing VDRE (VDRE-tk-CAT). In order to compare the activity of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ for transactivating the *VDRE* reporter gene, we selected monkey renal cancer cells (CV-1). These cells lack a functional VDR, so one can evaluate the binding activity of vitamin D analogues only in the transiently transfected VDR. The active metabolite of vitamin D, $1,25(OH)_2D_3$, should not show any increase in CAT activity if the cells are transfected only with VDRE-tk-CAT. As shown in Fig. 4, neither vitamin D_3 nor vitamin D_5 analogues could induce CAT activity, indicating a lack of endogenous VDR in these cells. However, when 500 ng VDR (Fig. 4b) was co-transfected with VDRE and the cells were incubated with 10 or 100 nM of $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$, there was enhanced expression of the *CAT* reporter gene. These results clearly indicate that both analogues of vitamin D can bind to the VDR and the complex can bind to the VDRE to initiate signal transduction. However, the extent of VDRE-reporter transactivation was 7- to 8-fold greater when the transfected cells were incubated with $1,25(OH)_2D_3$ at 10 nM and nearly 2-fold greater at 100 nM, respectively, compared with $1\alpha(OH)D_5$ at the same concentrations. This is consistent with the observed

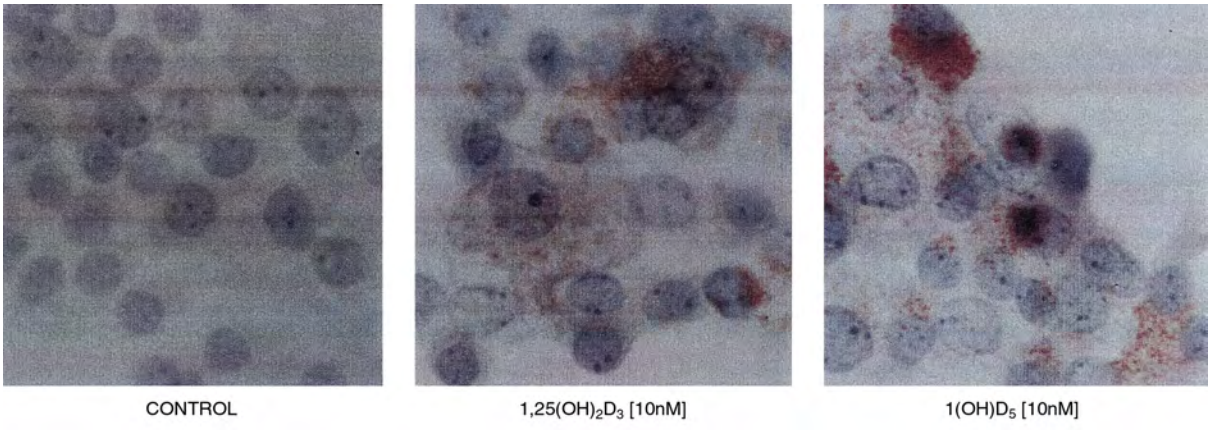

Fig. 3. Effects of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on lipid expression in T47D cells. A lipid assay was carried out as previously described in the presence or absence of vitamin D analogues.

finding that a log molar higher concentration of $1(OH)D_5$ is needed to obtain an equivalent response to that observed with $1,25(OH)_2D_3$.

3.4. Induction of VDR mRNA as determined by RT-PCR

Experiments were carried out to determine if VDR mRNA is induced by the vitamin D analogues in T47D and $MCF10_{neo}$ cells. Total RNA from the cells was isolated and reverse-transcribed. The cDNA was amplified using Taq polymerase and separated on 1.5% agarose gel. As shown in Fig. 5, the housekeeping gene *G3PDH* (C) was identical for all the cDNAs, indicating an equal loading of the gels. The VDR separated as a 420 bp fragment on the gel. As shown in Fig. 5(a), in T47D cells, there was a basal level of expression of VDR; however, incubation of cells for 3 days with either 10 or 100 nM of $1,25(OH)_2D_3$ increased the VDR expression in a dose-related manner. Similar results were also obtained with $1\alpha(OH)D_5$, as shown in Fig. 5(a). In contrast, $MCF10_{neo}$ cells expressed the basal level of VDR in the cells; but, there was no induction of VDR message by the vitamin D analogues (Fig. 5b). These results indicate that the lack of induction of differentiation by vitamin D in $MCF10_{neo}$ cells may be related to a lack of induction of VDR in these cells by vitamin D analogues.

4. Discussion

The effects of vitamin D analogues as differentiating agents and inhibitors of cell proliferation for breast cancer cells have been reported [1,7]. It is generally believed that the cells expressing VDR often respond to vitamin D analogues, whereas cells such as MDA-MB-231, which are ER- and express low or non-detectable

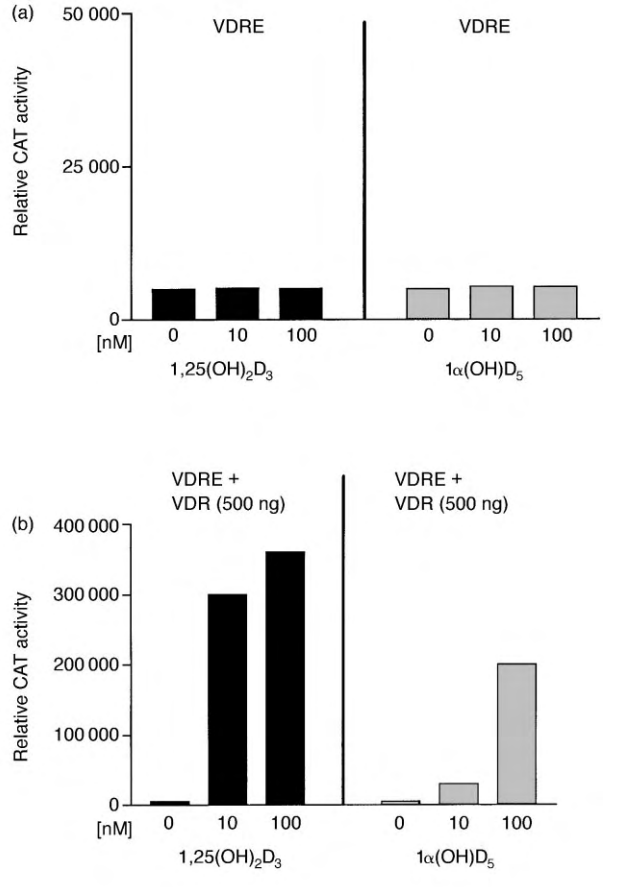

Fig. 4. Effects of $1,25(OH)D_3$ and $1\alpha(OH)D_5$ on the transactivation of the VDRE-conjugated reporter gene. Transient transfections of CV-1 cells with either VDRE-tk-CAT alone (a) or with VDR (b) was carried out by the calcium phosphate precipitation procedure. The cells were incubated with 10 and 100 nM vitamin D analogues for 3 days. CAT activity was measured spectrophotometrically. The experiments were repeated twice.

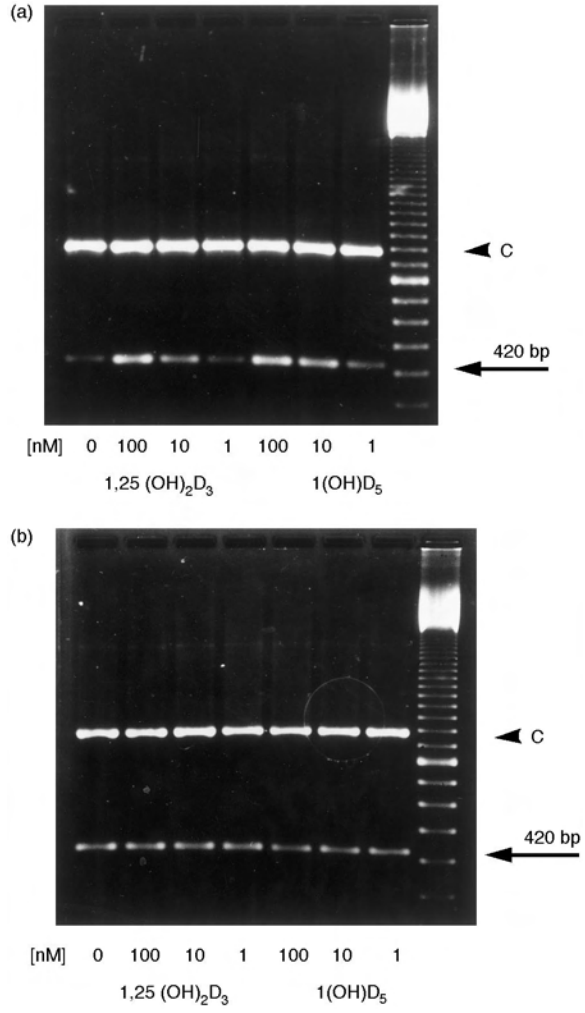

Fig. 5. Effects of vitamin D analogues on the expression of VDR mRNA in $MCF10_{neo}$ and T47D cells. Cells were incubated with various concentrations of analogues for 3 days in culture as previously described. VDR expression was measured by RT-PCR in (a) T47D cells; (b) $MCF10_{neo}$ cells. C, control housekeeping gene.

levels of VDR, do not respond to active vitamin D analogue(s) [24]. The VDR-mediated transcription regulatory genes include *TGFβ*, *EGF*, *c-myc* [25,26], and cell cycle regulators. The effects of various vitamin D analogues on programmed cell death have been evaluated in a variety of breast cancer cell lines. Consistently, MCF-7 cells which are ER+, VDR+ and positive for wild-type p53 exhibit apoptosis in response to vitamin D [27,28]. Although considerable literature exists for vitamin D-induced differentiation, its clinical application has been limited. This is due to its cytotoxicity at the concentration that induces differentiation. To this end, we have identified an analogue of the vitamin D_5 series which is non-calcaemic at the concentration at which 1,25-dihydroxyvitamin D_3 would induce hypercalcaemia. We previously reported that 1α-hydroxyvitamin D_5 inhibits carcinogen-induced development of mammary lesions in culture [12]. We also reported that it induces VDR and TGFβ in mammary epithelial cells. In this report, we addressed the question, ''Does 1-hydroxyvitamin D_5 induce cell differentiation of breast cancer cells to the same extent as the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3?''. T47D and $MCF10_{neo}$ cells were selected for the present study, since T47D cells are ER- and progesterone (PR)-positive and $MCF10_{neo}$ cells are negative for both ER and PR. Both analogues of vitamin D, $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, inhibited cell proliferation to the same extent and induced differentiation as determined by the increased expression of differentiation markers.

The $MCF10_{neo}$ cells were originally derived from normal breast tissue and the epithelial cells were subsequently immortalised. The $MCF10_{neo}$ cells are ER-VDR+ and stably transfected with ras. The cells are tumorigenic in athymic mice. Since both T47D and $MCF10_{neo}$ have similar VDR and p53 status and differ only in their ER status, we compared the response of T47D ER+ and $MCF10_{neo}$ cells to two analogues of vitamin D. The $MCF10_{neo}$ cells, like T47D cells, exhibited a suppression of cell proliferation; however, no induction of differentiation was noticed. This, therefore, raised the question of whether induction of VDR is essential for cell differentiation. We evaluated the induction of VDR mRNA by these two vitamin D analogues. The results showed that $MCF10_{neo}$ cells constitutively expressed VDR-mRNA. However, there was no induction of the VDR message by either of the vitamin D analogues. In contrast, there was a dose-dependent increase in the expression of VDR mRNA in the T47D cells by both vitamin D_3 and D_5 analogues. These results suggest that there may be a positive association between the differentiation of cells by vitamin D and the induction of vitamin D-induced mRNA of VDR. Alternatively, the antiproliferative effects may be mediated by p53 although this is most unlikely in this case as both $MCF10_{neo}$ and T47D cells do not have functional p53 [10] and yet they respond to antiproliferative activity of vitamin D analogues. These results suggest that the antiproliferative effects and differentiating effects of vitamin D analogues may be independent of the cellular p53 status. These results are consistent with a recent report indicating the non-involvement of p53 in vitamin D-mediated differentiating/cell growth suppressing functions in breast cancer cells. Thus, it is not clear what mechanism may be operative for the suppression of cell growth by vitamin D analogues. If both antiproliferative effects and cell differentiating effects are mediated by VDR, then it is possible that the constitutive level of VDR will be sufficient to mediate vitamin D's effects in suppressing cell proliferation but that induction of new VDR mRNA may be necessary for cell differentiation.

Comparison between the action of a natural ligand of vitamin D, $1,25(OH_2)D_3$, and a vitamin D_5 analogue was also made in terms of their ability to transactivate a VDRE-reporter *CAT* gene. We selected VDR-negative CV-1 cells for these studies so that the endogenous VDR would not interfere with the interpretation of data. Since CV-1 cells are truly VDR-negative, they do not respond to incubation with $1,25(OH_2)D_3$ and do not transactivate the VDRE-CAT reporter. Since both T47D and $MCF10_{neo}$ cells express basal levels, to different extents, of VDR, the vitamin D analogue-induced transactivation of the CAT reporter may vary between these two cells and will compromise comparing the two analogues of vitamin D. Results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ bind VDR and interact with VDRE. It was noted that, with 500 ng VDR transfection into CV-1 cells, $1,25(OH)D_3$ at 10 nM induced the reporter expression by more than 150-fold compared with the induction by $1\alpha(OH)D_5$ at the same concentration. The results indicate that, at equimolar concentrations, $1,25(OH)_2D_3$ is more potent in transactivating the *VDRE* reporter gene than $1(OH)D_5$. This is consistent with the earlier findings that, in mouse mammary gland organ cultures, the D_5 analogue is required at a log molar higher concentration to achieve similar effects to those observed with $1,25(OH)_2D_3$. The advantage, however, is that the D_5 analogue does not induce unwarranted toxicity which is often associated with 1,25-dihydroxyvitamin D_3. These studies collectively indicate that the vitamin D_5 series of agents mediate their action via the same VDR-mediated mechanism that is operative with the active metabolite of vitamin D_3.

Acknowledgements

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Abstract

The role of the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), in cell differentiation is well established. However, its use as a differentiating agent in a clinical setting is precluded due to its hypercalcaemic activity. Recently, we synthesised a relatively non-calcaemic analogue of vitamin D_5, 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), which inhibited the development of carcinogen-induced mammary lesions in culture and suppressed the incidence of chemically induced mammary carcinogmas in rats. In the present study, we determined the differentiating effects of 1α-$(OH)D_5$ in T47D human breast cancer cells and compared its effects with $1,25(OH)_2D_3$. Cells incubated with either 10 or 100 nM of the analogues inhibited cell proliferation in a dose-dependent manner, as measured by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. Similar growth-inhibitory effects were also observed for $MCF10_{neo}$ cells. Both vitamin D analogues induced cell differentiation, as determined by induction of casein expression and lipid production. However, $MCF10_{neo}$ cells failed to respond to either vitamin D analogue and did not undergo cell differentiation. Since the cell differentiating effect of vitamin D is considered to be mediated via the vitamin D receptor (VDR), we examined the induction of VDR using reverse transcriptase–polymerase chain reaction (RT-PCR) in both cells. The results showed that, in T47D cells, both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ induced VDR in a dose-dependent manner. Moreover, both analogues of vitamin D upregulated the expression of vitamin D response element-chloramphenicol acetyl transferase (VDRE-CAT). These results collectively indicate that 1α-$(OH)D_5$ may mediate its cell-differentiating action via VDR in a manner similar to that of $1,25(OH)_2D_3$.

Keywords: Vitamin D; Breast cancer cells; Differentiation; T47D; MCF10

1. Introduction

The research on vitamin D_3 and related compounds is currently at its apex. A vast amount of evidence has been collected, implicating the essential involvement of vitamin D metabolites in several cellular processes. The active metabolite 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) and related compounds suppress the development and progression of breast cancer and other carcinomas *in vivo* [1,2], inhibit the metastatic spread of tumour cells [3–5], and promote differentiation of breast cancer cells [6–8]. However, the calcaemic side-effects of $1,25(OH)_2D_3$ have prevented its application as a pharmaceutical agent. In recent years, considerable attention has been given to the development of vitamin D_3 analogues capable of inducing cell differentiation without systemic hypercalcaemia [8–10]. Many structural modifications are known to enhance several-fold the differentiating potency of vitamin D_3 analogues in normal (usually keratinocyte) or malignant (usually leukaemia) cell lines. Little attempt, however, has been made to evaluate vitamin D analogues of other series such as vitamin D_2, D_4, D_5 and D_6. This structural classification is based on the differences encountered in the side chain. Earlier studies reported that vitamin D_5 was the least toxic of vitamins D_2 through to D_6 [11].

During the past 2 years, we have been studying the role of 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), an analogue of vitamin D_5 (24-ethyl-vitamin D_3), on breast cancer cell differentiation. We have characterised its calcaemic

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activity in vitamin D-deficient Sprague–Dawley rats [12]. The analogue $1\alpha(OH)D_5$ was synthesised from sitosterol acetate and was found to be less calcaemic than vitamin D_3. It was observed that $1\alpha(OH)D_5$ was effective against the development of carcinogen-induced mammary lesions in mouse mammary gland organ cultures [12]. In a more recent study, we observed that $1\alpha(OH)D_5$ inhibited incidence and tumour multiplicity of N-methyl-N-nitrosourea-induced mammary adenocarcinoma in rats (data not shown). These results clearly demonstrate that this vitamin D analogue might be a good candidate in the prevention of mammary carcinogenesis. In the present study, we evaluated the effects of $1\alpha(OH)D_5$ on cell differentiation and proliferation in oestrogen receptor (ER)-positive T47D breast cancer cells and compared the effects of the D_5 analogue with the active metabolite of vitamin D_3, $1,25(OH)_2D_3$. Moreover, we compared the effects of vitamin D analogues between ER+ T47D cells and ER- $MCF10_{neo}$ cells. Both cell lines are negative for functional p53 [13].

It is well known that the nuclear activity of vitamin D_3 is based on the interaction of the vitamin D active metabolite, $1,25(OH)_2D_3$, with the vitamin D receptor (VDR) [14]. The VDR is a nuclear receptor that belongs to the superfamily of ligand-dependent transcription factors and is expressed in all the vitamin D target tissues. VDR mediates its action by conjugating with the Retinoid X Receptor (RXR) [15–17]. The VDR-RXR dimer, once formed, is capable of recognising the vitamin D response element (VDRE) in the promoter region of the gene. The VDRE is composed of direct repeats of 6 DNA bases separated by 3-base intervening sequences [18]. Vitamin D appears to play an important role in stabilising and transactivating the VDR/RXR–VDRE complex [19,20]. Its interaction with VDR, therefore, represents the central step in the transmission of a signal to the transcription machinery, resulting in activation or suppression of transcription of genes leading ultimately to differentiation. We recently showed that the normal human breast epithelial cells lacking functional VDR do not respond to vitamin D to induce cell differentiation. However, transient transfection of VDR in these HBL-100 cells resulted in increased association of VDR-VDRE, as measured by the CAT reporter assay [21]. In the present study, we compared the effects of $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ on the transactivation of VDR–VDRE in T47D cells.

2. Materials and methods

2.1. Cells

The breast epithelial cell line, $MCF10_{neo}$, and human breast cancer cell line, T47D, were obtained from the American Type Culture Collection (Rockville, MD, USA). The $MCF10_{neo}$ cells were maintained in minimum essential medium with Earl's salts (MEME) medium supplemented with 10% fetal bovine serum (FBS), whereas T47D cells were maintained in RPMI supplemented with 0.2 I.U. bovine insulin/ml and 10% FBS. The monkey renal cancer CV-1 cells were maintained in MEME with 10% FBS supplement.

2.2. MTT assay

The cells were seeded in a 96-well/plate at a density of 500 cells/well in 100 μl/well of cell culture medium supplemented with 10% steroid-stripped serum. 24 h after seeding, the cells were incubated with 10 and 100 nM concentrations of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, respectively. The medium was changed every 3 days. After 7 days, the cultures were used for the dimethylthiazolyl-2,5-diphenyltetrazolium Bromide (MTT) assay. MTT (5 mg/ml in phosphate buffered serum (PBS)) was added to the wells (15 μl/well) and incubated at 37°C for 2 h. The stop solution (20% sodium dodecyl sulphate (SDS) in 50% N,N-dimethylformamide) was then added (100 μl/well) and incubated for an additional 2 h. The plates were scanned at 590 nm OD, and the results for each treatment group were averaged.

2.3. Immunohistochemistry

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS and fixed in 10% formalin for 5 min. The fixed cells were further incubated in cold methanol for 3 min and acetone for 2 min. After blocking the cells with a protein block/normal goat serum (BioGenex, San Ramos, CA, USA), they were incubated with casein antibody (100 μg/ml) (Accurate Chemical and Scientific Corp. Westbury, NY, USA) for 2 h. The cells were then incubated with secondary anti-mouse biotinylated antibody for 30 min, followed by streptavidin–peroxidase complex and 3,3′-diaminobenzidine (DAB) solution as chromogen. Appropriate controls were performed to rule out non-specific staining with secondary antibody.

2.4. Lipid assay

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS, and fixed by incubating in cold methanol for 3 min and propylene glycol for 2 min. The cells were, at this point, stained with Oil Red O' for 30 min and rinsed in isopropyl alcohol then de-ionised water. Haematoxylene staining for 30 s and Scott solution rinse completed the assay.

2.5. RNA isolation and RT-PCR

The cells were incubated with 1, 10 or 100 nM $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 3 days. The medium

from the tissue culture flasks was removed and the cells were treated with RNA-Zol B (Tel-Test Inc., Friendswood, TX, USA). RNA were isolated according to the manufacturer's instruction and quantified spectrophotometrically. Reverse transcription (RT) and PCR were carried out using Advantage RT for PCR and Advantage cDNA PCR kit (Clontech Inc., Palo Alto, CA, USA). Primer sequences for VDR were selected and custom-synthesised by Oligos Etc. The sense primer was 5′-GGA GTT GCT GTT TGT TTG AC, and the antisense primer was 5′-CTT CTG TGA GGC TGT TTT TG. The primer for the housekeeping gene *G3PDH* was purchased from ClonTech. The touchdown PCR procedure was employed with minor modifications [22]. The first strand cDNA was heated at 94°C for 1 min followed by denaturation at 94°C for 45 sec, annealing at 68–66–64–62–60°C for 45 sec each time and extension at 72°C for 2 min, for 26 cycles. The final cycle was followed by a 7-min extension step at 72°C to ensure that the amplified DNA was double stranded. The absence of contaminant was routinely checked by RT-PCR assays of negative control samples (sterile buffer, provided in the kit). The PCR products were separated on 1.5% agarose gel at 64 volts for 3 h, stained with ethidium bromide and visualised by ultraviolet (UV)-transillumination.

2.6. Transient transfection

The reporter construct VDRE-tk-CAT was prepared by inserting a copy of VDRE into the *Bam*HI site of the pBLCAT$_2$ as previously described [23]. For transfection, 1×10^5 CV-1 cells were plated in 24-well plates. Transfections were carried out using the calcium phosphate precipitation procedure. Briefly, 100 ng of VDRE-tk-CAT reporter plasmid, 250 ng of β-galactosidase (β-gal) expression vector, and 500 ng of VDR expression vectors were mixed with carrier DNA (pBluescript) to 1 μg of total DNA per well. The CAT activity was normalised for transfection efficiency by the corresponding β-gal activity.

3. Results

3.1. Effect of 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ on cell proliferation

The breast epithelial cells MCF10$_{neo}$ and breast cancer cells T47D were incubated with the vitamin D analogues for 7 days in culture. After this, the effects of vitamin D analogues were evaluated by the MTT assay. The results indicated a 31% and 50% growth inhibition for MCF10$_{neo}$ at 10 and 100 nM of 1,25(OH)$_2$D$_3$ concentrations, respectively, as compared with 50% and 72% inhibition with 1α(OH)D$_5$ at 10 and 100 nM, respectively (Fig. 1a). The ER-positive, T47D cells showed a 29% and 52.5% growth inhibition after being exposed for 7 days to 1,25(OH)$_2$D$_3$ at 10 and 100 nM, respectively. Unlike MCF10$_{neo}$ cells, T47D cells did not exhibit increased growth suppression when exposed to 1α(OH)D$_5$. Both analogues suppressed growth of T47D cells by approximately 30% and 50% at low and high concentrations, respectively (Fig. 1b). These results suggest that both 1α(OH)D$_5$ and 1,25(OH)$_2$D$_3$ are comparable in producing antiproliferative effects in breast cancer cells.

3.2. Induction of differentiation of breast cancer cell lines

Since one of the major recognised functions of vitamin D is induction of cell differentiation, we evaluated the effects of both analogues on the induction of differentiation in both cell lines. As markers of cell differentiation, we used casein and lipid. Casein expression was measured by immunocytochemistry using casein antibodies. Results showed that, for T47D cells, casein was expressed in less than 10% of the control cells.

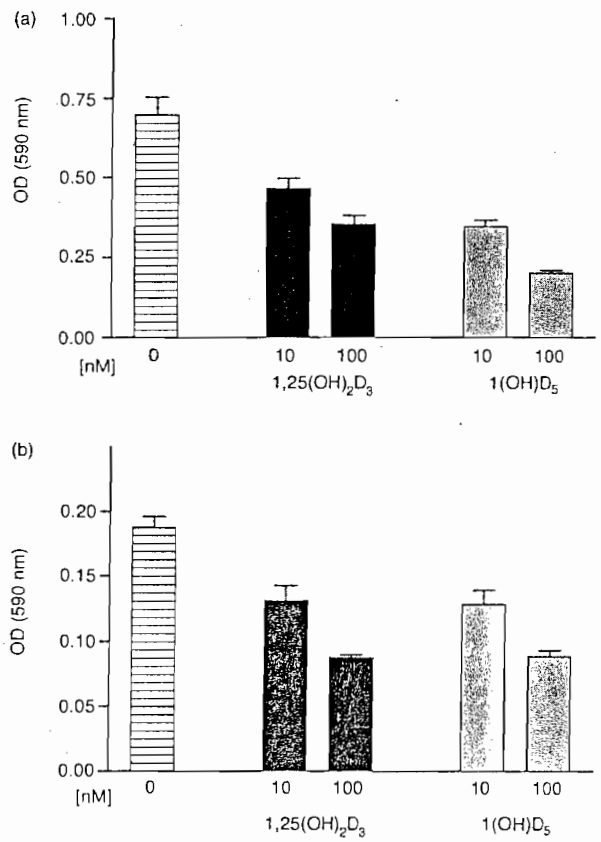

Fig. 1. Effects of 1,25(OH)$_2$D$_3$ and 1α (OH)D$_5$ on the proliferation of MCF10$_{neo}$ cells and T47D cells. The MTT assay was carried out using duplicate cultures and the experiments were repeated three times. The error bars represent the standard deviation. (a) MCF10$_{neo}$ cells; (b) T47D cells.

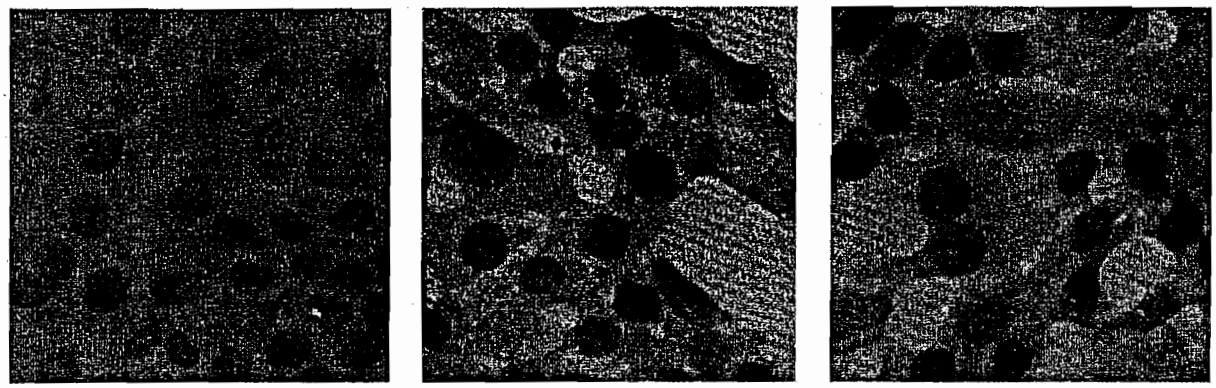

Fig. 2. Effect of vitamin D analogues on casein expression in T47D cells. Immunohistochemical staining for casein expression was carried out as previously described in the presence or absence of the vitamin D analogues.

After 7 days treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, the intensity and number of cells expressing casein increased to approximately 70 and 85% at 10 and 100 nM concentrations, respectively. No difference was noticed between the effects of D_3 or D_5 analogues (Fig. 2 and data not shown). Similarly, there was a dramatic increase in the expression of lipid production in T47D cells after 7 days of treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ (Fig. 3). These results indicated that both vitamin D analogues induce cell differentiation in T47D cells. In contrast, the $MCF10_{neo}$ cells, tested for the same markers of differentiation, did not show any presence or induction of either casein or lipids in the control cells or in cells exposed to vitamin D_3 or D_5 (data not shown).

3.3. Transactivation of VDRE

The VDRE transactivation activity of the vitamin D analogues was determined using the *CAT* reporter gene containing VDRE (VDRE-tk-CAT). In order to compare the activity of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ for transactivating the *VDRE* reporter gene, we selected monkey renal cancer cells (CV-1). These cells lack a functional VDR, so one can evaluate the binding activity of vitamin D analogues only in the transiently transfected VDR. The active metabolite of vitamin D, $1,25(OH)_2D_3$, should not show any increase in CAT activity if the cells are transfected only with VDRE-tk-CAT. As shown in Fig. 4, neither vitamin D_3 nor vitamin D_5 analogues could induce CAT activity, indicating a lack of endogenous VDR in these cells. However, when 500 ng VDR (Fig. 4b) was co-transfected with VDRE and the cells were incubated with 10 or 100 nM of $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$, there was enhanced expression of the *CAT* reporter gene. These results clearly indicate that both analogues of vitamin D can bind to the VDR and the complex can bind to the VDRE to initiate signal transduction. However, the extent of VDRE-reporter transactivation was 7- to 8-fold greater when the transfected cells were incubated with $1,25(OH)_2D_3$ at 10 nM and nearly 2-fold greater at 100 nM, respectively, compared with $1\alpha(OH)D_5$ at the same concentrations. This is consistent with the observed

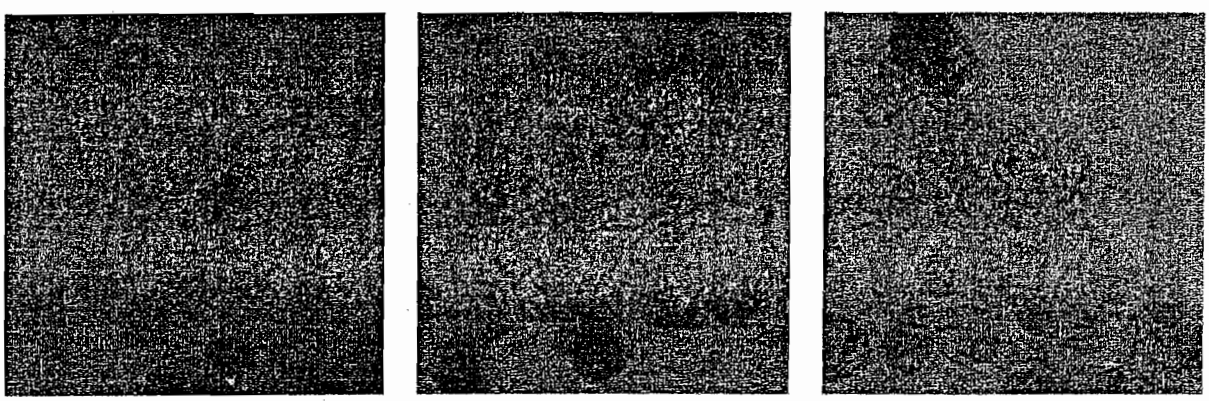

Fig. 3. Effects of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on lipid expression in T47D cells. A lipid assay was carried out as previously described in the presence or absence of vitamin D analogues.

finding that a log molar higher concentration of $1(OH)D_5$ is needed to obtain an equivalent response to that observed with $1,25(OH)_2D_3$.

3.4. Induction of VDR mRNA as determined by RT-PCR

Experiments were carried out to determine if VDR mRNA is induced by the vitamin D analogues in T47D and $MCF10_{neo}$ cells. Total RNA from the cells was isolated and reverse-transcribed. The cDNA was amplified using Taq polymerase and separated on 1.5% agarose gel. As shown in Fig. 5, the housekeeping gene *G3PDH* (C) was identical for all the cDNAs, indicating an equal loading of the gels. The VDR separated as a 420 bp fragment on the gel. As shown in Fig. 5(a), in T47D cells, there was a basal level of expression of VDR; however, incubation of cells for 3 days with either 10 or 100 nM of $1,25(OH)_2D_3$ increased the VDR expression in a dose-related manner. Similar results were also obtained with $1\alpha(OH)D_5$, as shown in Fig. 5(a). In contrast, $MCF10_{neo}$ cells expressed the basal level of VDR in the cells; but, there was no induction of VDR message by the vitamin D analogues (Fig. 5b). These results indicate that the lack of induction of differentiation by vitamin D in $MCF10_{neo}$ cells may be related to a lack of induction of VDR in these cells by vitamin D analogues.

4. Discussion

The effects of vitamin D analogues as differentiating agents and inhibitors of cell proliferation for breast cancer cells have been reported [1,7]. It is generally believed that the cells expressing VDR often respond to vitamin D analogues, whereas cells such as MDA-MB-231, which are ER- and express low or non-detectable

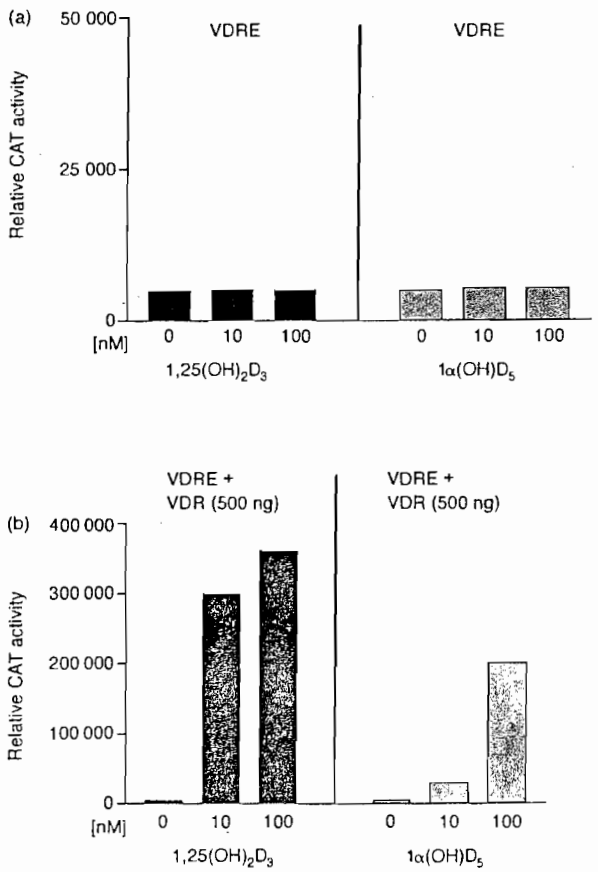

Fig. 4. Effects of $1,25(OH)D_3$ and $1\alpha(OH)D_5$ on the transactivation of the VDRE-conjugated reporter gene. Transient transfections of CV-1 cells with either VDRE-tk-CAT alone (a) or with VDR (b) was carried out by the calcium phosphate precipitation procedure. The cells were incubated with 10 and 100 nM vitamin D analogues for 3 days. CAT activity was measured spectrophotometrically. The experiments were repeated twice.

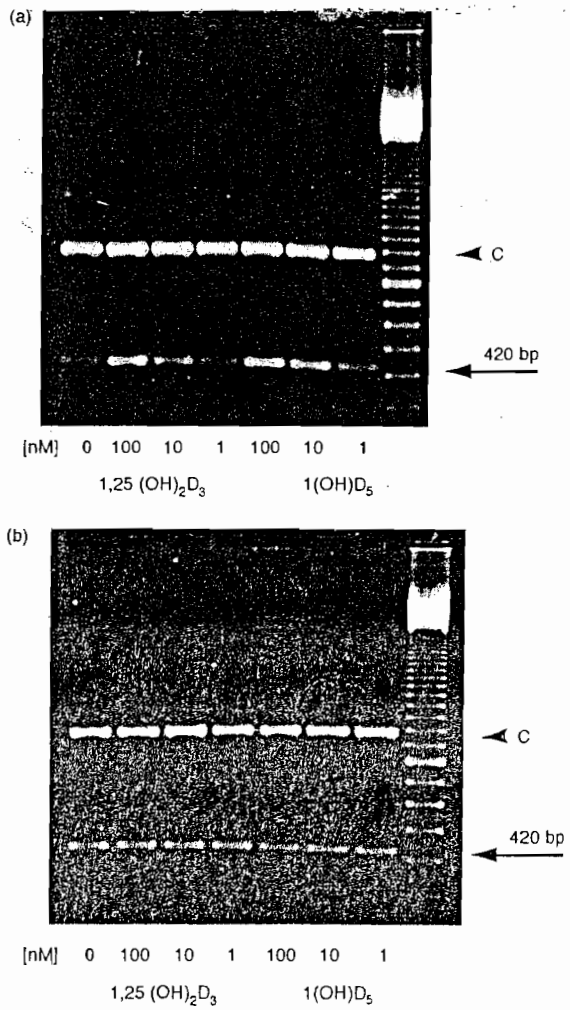

Fig. 5. Effects of vitamin D analogues on the expression of VDR mRNA in $MCF10_{neo}$ and T47D cells. Cells were incubated with various concentrations of analogues for 3 days in culture as previously described. VDR expression was measured by RT-PCR in (a) T47D cells; (b) $MCF10_{neo}$ cells. C, control housekeeping gene.

levels of VDR, do not respond to active vitamin D analogue(s) [24]. The VDR-mediated transcription regulatory genes include *TGFβ*, *EGF*, *c-myc* [25,26], and cell cycle regulators. The effects of various vitamin D analogues on programmed cell death have been evaluated in a variety of breast cancer cell lines. Consistently, MCF-7 cells which are ER+, VDR+ and positive for wild-type p53 exhibit apoptosis in response to vitamin D [27,28]. Although considerable literature exists for vitamin D-induced differentiation, its clinical application has been limited. This is due to its cytotoxicity at the concentration that induces differentiation. To this end, we have identified an analogue of the vitamin D_5 series which is non-calcaemic at the concentration at which 1,25-dihydroxyvitamin D_3 would induce hypercalcaemia. We previously reported that 1α-hydroxyvitamin D_5 inhibits carcinogen-induced development of mammary lesions in culture [12]. We also reported that it induces VDR and TGFβ in mammary epithelial cells. In this report, we addressed the question, "Does 1-hydroxyvitamin D_5 induce cell differentiation of breast cancer cells to the same extent as the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3?". T47D and $MCF10_{neo}$ cells were selected for the present study, since T47D cells are ER- and progesterone (PR)-positive and $MCF10_{neo}$ cells are negative for both ER and PR. Both analogues of vitamin D, $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, inhibited cell proliferation to the same extent and induced differentiation as determined by the increased expression of differentiation markers.

The $MCF10_{neo}$ cells were originally derived from normal breast tissue and the epithelial cells were subsequently immortalised. The $MCF10_{neo}$ cells are ER-VDR+ and stably transfected with ras. The cells are tumorigenic in athymic mice. Since both T47D and $MCF10_{neo}$ have similar VDR and p53 status and differ only in their ER status, we compared the response of T47D ER+ and $MCF10_{neo}$ cells to two analogues of vitamin D. The $MCF10_{neo}$ cells, like T47D cells, exhibited a suppression of cell proliferation; however, no induction of differentiation was noticed. This, therefore, raised the question of whether induction of VDR is essential for cell differentiation. We evaluated the induction of VDR mRNA by these two vitamin D analogues. The results showed that $MCF10_{neo}$ cells constitutively expressed VDR-mRNA. However, there was no induction of the VDR message by either of the vitamin D analogues. In contrast, there was a dose-dependent increase in the expression of VDR mRNA in the T47D cells by both vitamin D_3 and D_5 analogues. These results suggest that there may be a positive association between the differentiation of cells by vitamin D and the induction of vitamin D-induced mRNA of VDR. Alternatively, the antiproliferative effects may be mediated by p53 although this is most unlikely in this case as both $MCF10_{neo}$ and T47D cells do not have functional p53 [10] and yet they respond to antiproliferative activity of vitamin D analogues. These results suggest that the antiproliferative effects and differentiating effects of vitamin D analogues may be independent of the cellular p53 status. These results are consistent with a recent report indicating the non-involvement of p53 in vitamin D-mediated differentiating/cell growth suppressing functions in breast cancer cells. Thus, it is not clear what mechanism may be operative for the suppression of cell growth by vitamin D analogues. If both antiproliferative effects and cell differentiating effects are mediated by VDR, then it is possible that the constitutive level of VDR will be sufficient to mediate vitamin D's effects in suppressing cell proliferation but that induction of new VDR mRNA may be necessary for cell differentiation.

Comparison between the action of a natural ligand of vitamin D, $1,25(OH_2)D_3$, and a vitamin D_5 analogue was also made in terms of their ability to transactivate a VDRE-reporter *CAT* gene. We selected VDR-negative CV-1 cells for these studies so that the endogenous VDR would not interfere with the interpretation of data. Since CV-1 cells are truly VDR-negative, they do not respond to incubation with $1,25(OH_2)D_3$ and do not transactivate the VDRE-CAT reporter. Since both T47D and $MCF10_{neo}$ cells express basal levels, to different extents, of VDR, the vitamin D analogue-induced transactivation of the CAT reporter may vary between these two cells and will compromise comparing the two analogues of vitamin D. Results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ bind VDR and interact with VDRE. It was noted that, with 500 ng VDR transfection into CV-1 cells, $1,25(OH)D_3$ at 10 nM induced the reporter expression by more than 150-fold compared with the induction by $1\alpha(OH)D_5$ at the same concentration. The results indicate that, at equimolar concentrations, $1,25(OH)_2D_3$ is more potent in transactivating the *VDRE* reporter gene than $1(OH)D_5$. This is consistent with the earlier findings that, in mouse mammary gland organ cultures, the D_5 analogue is required at a log molar higher concentration to achieve similar effects to those observed with $1,25(OH)_2D_3$. The advantage, however, is that the D_5 analogue does not induce unwarranted toxicity which is often associated with 1,25-dihydroxyvitamin D_3. These studies collectively indicate that the vitamin D_5 series of agents mediate their action via the same VDR-mediated mechanism that is operative with the active metabolite of vitamin D_3.

Acknowledgements

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Vitamin D and cancer

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Abstract

Vitamin D, a steroid hormone and exerts its biological effects through its active metabolite 1α, 25 dihydroxyvitamin D3 [1,25(OH)2D3]. Like steroid hormones, 1,25(OH)2D3 is efficacious at very low concentrations and serves as a ligand for vitamin D receptors (VDR), associating with VDR very high affinity. Despite its potent property as a differentiating agent, its use in the clinical practice is hampered by the induction of hypercalcemia at a concentration required to suppress cancer cell proliferation. Therefore nearly 400 structural analogs of vitamin D3 have been synthesized and evaluated for their efficacy and toxicity. Among these analogs, relatively less toxic but highly efficacious analogs, EB1089, RO24–5531, 1α-hydroxyvitamin D5 and a few others have been evaluated in a preclinical toxicity and in Phase I clinical trials for dose tolerance in advanced cancer patients. Clinical trials using vitamin D analogs for prevention or therapy of cancer patients are still in their infancy. Vitamin D mediates its action by two independent pathways. Genomic pathway involves nuclear VDR and induces biological effects by interactions with hormone response elements and modulation of differential gene expressions. Evidence also suggests that vitamin D analogs also interact with steroid hormone(s) inducible genes. The non-genomic pathway is characterized by rapid actions of vitamin D. It involves interactions with membrane-VDR interactions and its interactions with protein kinase C and by altering intracellular calcium channels. Thus, the development of nontoxic analogs of vitamin D analogs and understanding of their molecular mechanism(s) of action are of significant importance in the prevention and treatment of cancer by vitamin D.

Keywords: Vitamin D; Analogs of vitamin D; VDR; Carcinogenesis; Metabolism; Mechanism of action

1. Background

Vitamin D was discovered by Edward Mellanby in 1919 during his classic experiments with rickets [1]. It is a family of compounds consisting of 9,10 secosteroids, which differ, in their side-chain structures. They are classified into five forms [2]; vitamin D2, ergosterol; D3, cholecalciferol; D4, 22,23 dihydroergoalciferol; D5 sitosterol (24-ethylcholecalciferol) and D6 stigmasterol (Fig. 1). Vitamin D is derived from a cholesterol-like precursor, 7-dehydrocholesterol. When human skin is exposed to sunlight, the UV-B photons (between 290–315 nm) interact with 7-dehydrocholesterol causing photolysis and cleavage of the B-ring of the steroid structure, which upon thermoisomerization results into a secosteroid [3,4]. In order to produce physiological activity, vitamin D has to be metabolized. Numerous in-depth reviews focusing on the metabolism of vitamin D have been published. Since the metabolism of vitamin D is not the primary focus of this article, a simplistic overview of D-metabolism is briefly discussed here. The pro-hormone vitamin D gets metabolized to 25-hydroxyvitamin D in liver by 25-hydroxylase. This metabolite is present in the circulation at a concentration of more than 0.05 μM (20 ng/ml). The active metabolite of vitamin D, however is generated by hydroxylation of 25-hydroxyvitamin D at 1α-position in kidney. The enzyme 1α-hydroxylase has also been shown to be present in keratinocytes and prostate epithelial cells, suggesting that the fact that target organs may also be able to generate 1,25 dihydroxyvitamin D3 from 25-hydroxyvitamin D3 [5]. More recently mRNA for 25-hydroxvitamin D-1α-hydroxylase has been reported in normal and malignant colon tissue [6,7]. The active metabolite 1α,25-dihydroxyvitamin D is present in the human plasma at a concentration of 0.05–0.15 nM (20–60 pg/ml) [8,9]. In addition to 1α-hydroxylation of 25-hydroxyvitamin D3, many metabolites have been identified. These metabolites are side chain modifications with no definitive function assigned to them. The overall path of metabolism of vitamin D2 is similar to vitamin D3 with a few differences [10]. Both 25-hydroxyvitamin D2 and 1α,25-dihydroxyvitamin

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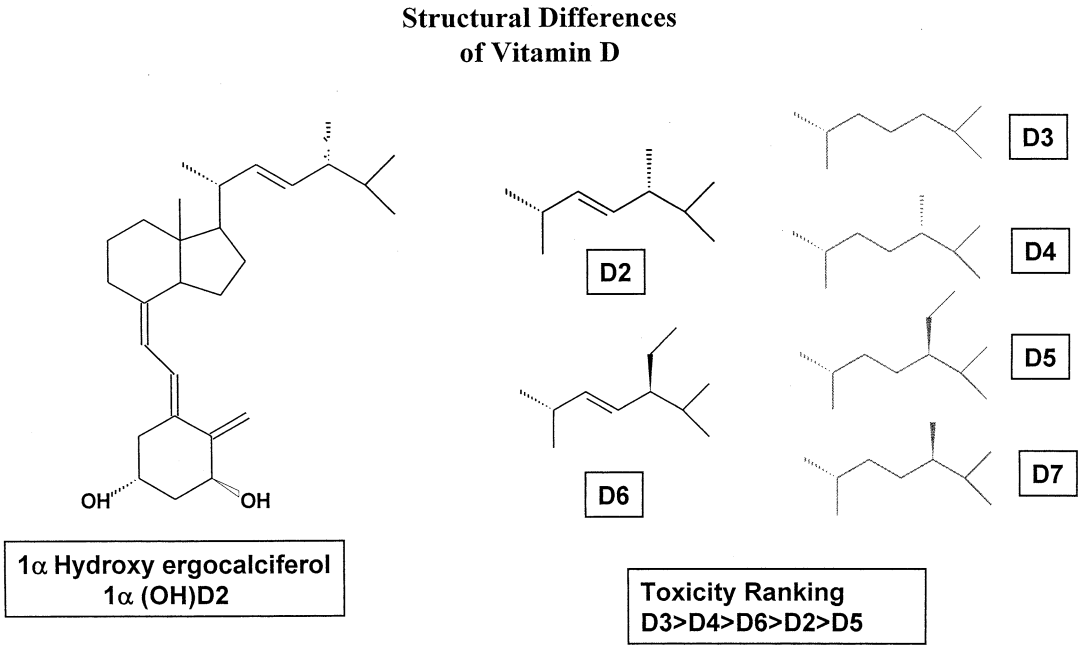

Fig. 1. Structural differences of vitamin D. Vitamin D has been classified into various classes of D2 through D7. Ergocalciferol is classified as vitamin D2 and vitamin D6 is a 24-ethyl analog of vitamin D2. On the other hand vitamin D3 or cholecalciferol is modified by either methyl or ethyl group on C-24 position. These vitamin D molecules are further classified as D3, D4, D5 and D7.

D2 have been evaluated for their biological functions. The catabolism of vitamin D occurs by further hydroxylation of 25-hydroxyvitamin D3 by 24 hydroxylase to yield 24,25 dihydroxyvitamin D3. The enzyme 24-hydroxylase is ubiquitous and is expressed in all the cells expressing vitamin D receptors (VDR). The enzyme is regulated by PTH and 1α,25-dihydroxyvitamin D3. The major significance of 24-hydroxylation is inactivation of vitamin D3 [11,12]. The inactivated vitamin D metabolites are nonfunctional. The overall metabolism of vitamin D is outlined in Fig. 2.

2. Experimental basis for vitamin D and cancer

For the past 20 years it has been consistently reported and well established that the active metabolite of vitamin D, 1,25(OH)2D3 exhibits potent cell differentiating property in leukemia cells as well as much cancer cells [13,14]. The antiproliferative and differentiation-inducing effects can be of clinical significance in prevention or treatment of cancer of several target organs. One of the main limitations in this modulation is the fact that the concentration required for being efficacious for 1,25(OH)2D3, is also very toxic. The effective concentration of 1,25(OH)2D3 induces dangerously high levels of serum calcium in experimental animals resulting in body weight loss and could be occasionally lethal [15]. This has resulted in the synthesis of analogs of vitamin D molecule with the hope of generating an analog that is effective in prevention of cancer or suppressing growth of cancer cells in culture and in vivo models without expressing any toxic adverse effects. Typically, the vitamin D structure is divided into four parts. The A ring, B ring, CD ring and the side chain. The alterations can be made at all these four sites, except the modification of the CD ring is not very common due to the rigid structure. The maximum alterations, on the other hand, are made from the open side chain. Nearly 400 analogs of vitamin D have been synthesized and many of them have been evaluated [16,17]. As far as the efficacy in in vitro or in vivo cancer models are concerned, where the risk benefit ratio related to toxicity and efficacy is determined, only a handful of vitamin D-chemicals have been successfully utilized [16,18]. The most widely studied analogs besides 1,25-dihydroxy D3, include 22-oxa-calcitriol [19,20] (Chugai Pharmaceuticals, Japan), EB1089 [21] (Leo Pharmaceuticals, Denmark), calcipotriol, KH1060 [22] (Leo Pharmaceuticals, Denmark), R024–5531 [23,24] (Hoffman la Roche, Nutley, NJ) and recently synthesized analog from our laboratory, 1α-hydroxy-24 ethyl-vitamin D3 [25] (1α(OH) D5, OncQuest, Chicago, IL). The side chain modifications of vitamin D3 molecule to result in these selective structures is shown in Fig. 3. All these analogs have been evaluated in a variety of cancer cell culture models, in vivo carcinogenesis models and in xenograft models using athymic mice. The main selection criteria here is to adopt a compound that does not induce hypercalcimia or other undesirable side effects at the effective dose level. The criteria for selection of vitamin D agents for other conditions such as bone disease, immunomodulation or hormonal therapy or nutrition can be very different and will not be discussed here since it will not be within the scope of this review.

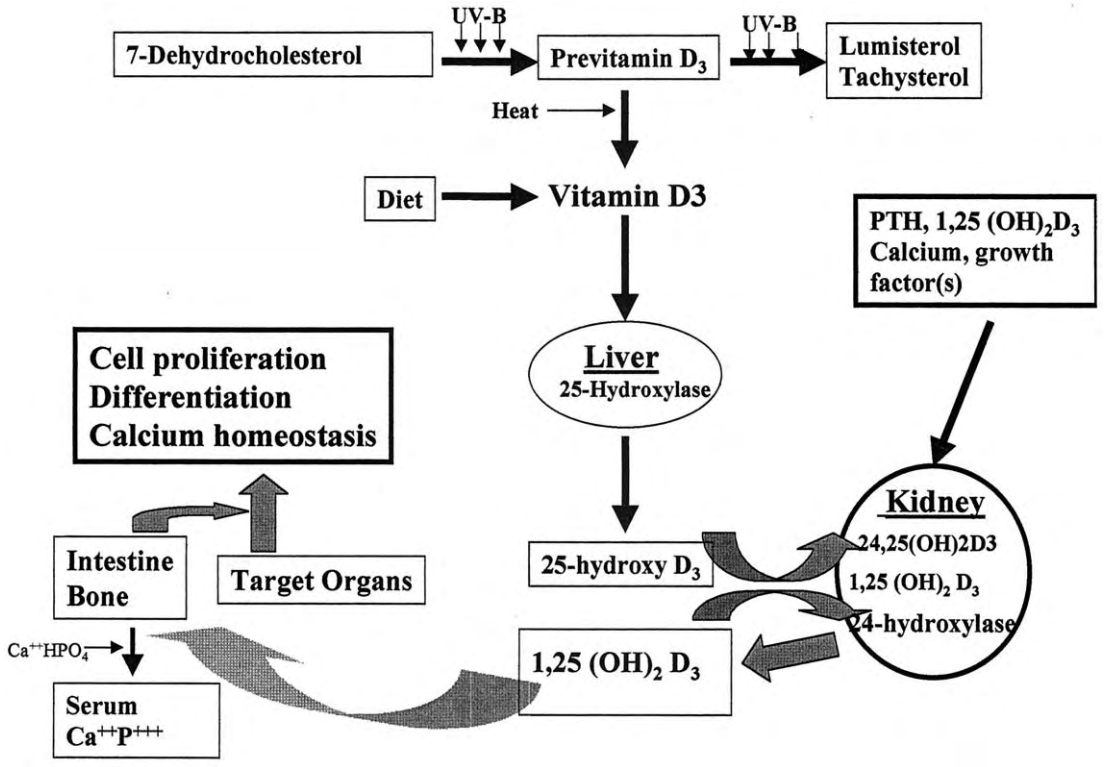

Fig. 2. Metabolism of vitamin D. Conversion of 7-hydrocholesterol to previtamin D3 by UV light and its subsequent processing to vitamin D3 and active metabolite 1,25-dihydroxyvitamin D3 is schematically shown. Vitamin D metabolism by liver and its processing by kidney is also shown in this diagram.

2.1. Efficacy of vitamin D analogs on breast cancer in vitro

Effects of vitamin D analogs on cell proliferation has been studied in a number of breast cancer cell lines as well as on the cells derived from many other target organs. The breast cancer cell lines expressing estrogen receptor (ER+) as well as ER-status have been utilized. All the analogs evaluated thus far have shown antiproliferative effects on ER+ breast cancer cells [26]. However, the effects of vitamin D on the ER- cells are not consistent. 1α(OH)D5 is effective against ER- BCA-4 cells whereas it is ineffective against ER-BCA1 and MDA-MB 231 and MDA-MB-468 cells [27,28,29]. The MDA-MB cell lines express vitamin D receptor poorly. Presence of low expression of VDR and absence of VDR in these cells has been reported. On the other hand, all ER+ cell lines express VDR and are responsive to vitamin D analogs (Table 1). We and others have shown that except for some ER+ breast cancer cells such as MCF-7 and BT474 cells, vitamin D analogs do not induce apoptosis [30,31]. The majority of the cells respond to vitamin D by induction of cell differentiation. Induction of cell difefrentiation is analyzed by cell morphology, flow cytometry, lipid expression and expression of casein and integrin α2 in breast cancer cells [27]. Table 1 summarizes the effects of all the commonly used analogs of vitamin D. The majority of the analogs showed efficacy against ER+ cells at noncalcemic concentrations that are greater than 1,25(OH)2D3. Among the agents effective against ER- cells, KH1060 and 22-oxa-calcitriol appeared to be very effective against MDA-MB-231. KH1060, however was not effective against MDA-MB-435. This is especially interesting since MDA-MB-231 cells are reported to have either no VDR or very low expression of VDR. Both KH1060 and 22-oxa-calcitriol have similar chemical alteration at C-22 position and both are effective against MDA-MB-231 cells. Thus may provide an altered mechanism of action that may not involve VDR or estrogen responsiveness. Other efficacious analogs including 1α(OH)D5 are not effective in VDR- breast cancer cells (Table 1).

In addition to cell culture models experiments have been carried out in mammary gland organ culture model. Mouse mammary gland responds to carcinogen in the presence of growth promoting hormones and form precancerous alveolar or ductal lesions [32]. It has been shown that transplantation of epithelial cells prepared from these glands form adenocarcinoma in syngeneic mice [33]. This model has been used for studying efficacy of chemopreventive agents and understanding mechanism of their action. Comparison of 1,25-dihydroxyvitamin D3, RO24–5531 and 1α-hydroxyvitamin D5 indicated that the D5 analog exhibited similar activity compared to dihydroxy D3 at a log molar higher concentration. RO24–5531 and EB 1089 were toxic at concentration higher than 1 μM [25], whereas 1α-hydroxyvitamin D5 can be tolerated at higher concentrations. The analog 1α(OH)D5 induced VDR and TGFβ in the

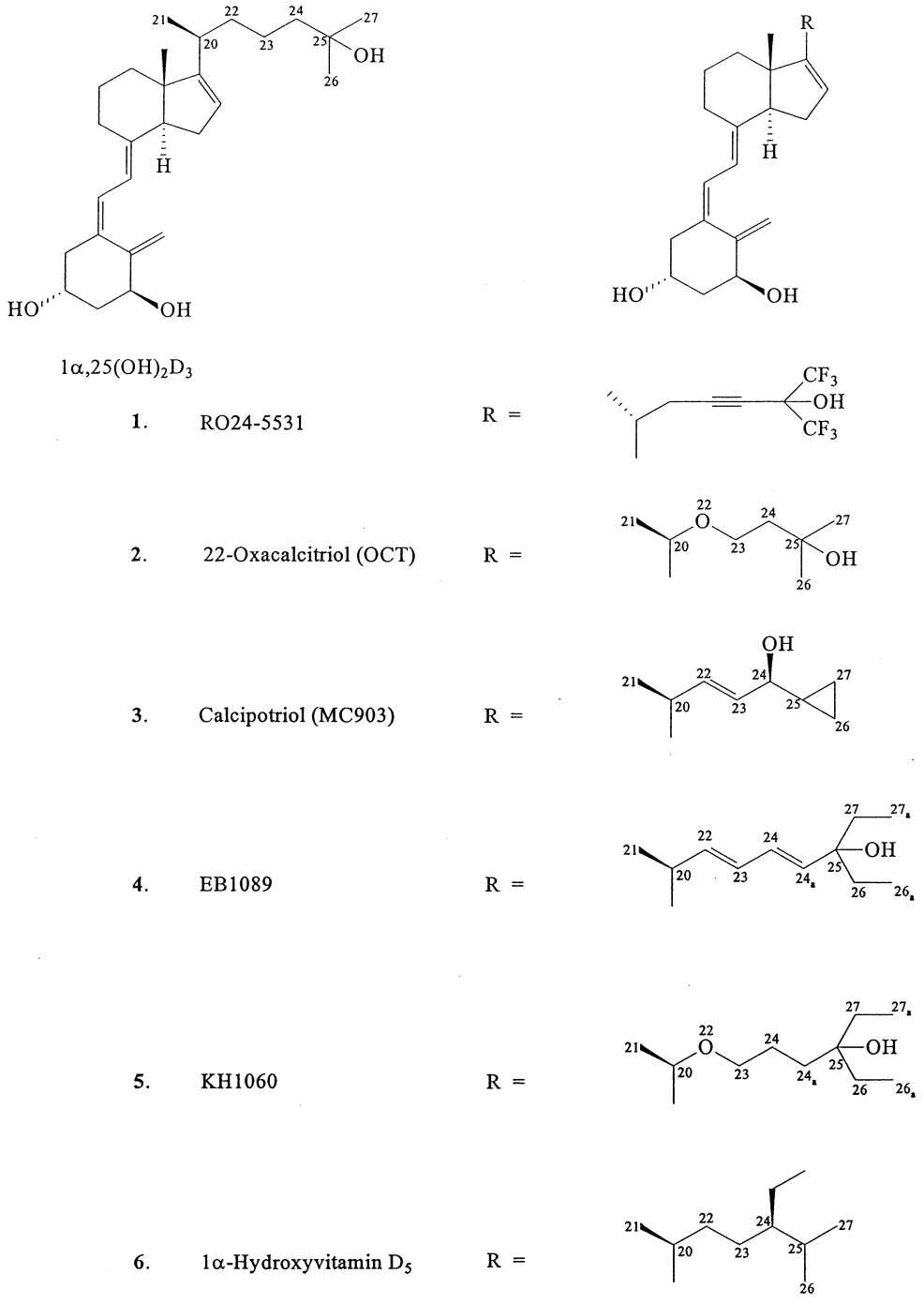

Fig. 3. Chemical structures of some of the active analogs of vitamin D.

mammary glands. These results suggested that the inhibitory effect of vitamin D analog 1α(OH)D5 be mediated by VDR.

2.2. Vitamin D and other cancers

Effects of vitamin D analogs have been evaluated in a number of cell types. The majority of cancer cell types, including HL60 leukemia, Coco and HT29 human colon cancer cells and a variety of prostate cancer cells including LnCap cells are all responsive to vitamin D analogs [34]. More recently it was noted that the incubation of prostate cancer cells as well as normal prostate epithelial cells express 1α-hydroxylase activity which is responsible for converting 25-hydroxyvitamin D3 to the active metabolite 1,25-dihydroxyvitamin D3 [35,36]. Since 25-hydroxyvitamin D3 is less calcemic and less toxic compared to the dihydroxyvitamin D3, it may be more suitable for prostate cancer prevention and therapy. Moreover, all prostate can-

Table 1
Summary of efficacy of vitamin D analogs in cancer cell prolifration

Target organ	Cells	Vitamin D analogs	Efficacy	Comments
Breast	ER+			
	MCF-7, ZR75-1, T47D BT474, BT20, SK-BR-3	22-oxa-calcitriol, 1α(OH)D_5EB-1089, KH11060, MC903, RO24-5531, 22-oxa-Calcitriol	All effective	VDR+
	ER-			
	MDA-MB-231, MDA-MB-436	1α-(OH)D_5, 22-oxa-calcitriol, KH(1060, RO24-5531	Ineffective	VDR+/−
	UISO-BCA-4	1α(OH)D_5	Effective	VDR+
	UISO-BCA-1	1α-(OH)D_5	Ineffective	VDR−
	MDA-MB-231,	22-oxa-calcitriol	Effective	VDR+/−
Prostate	LnCap, PC-3	1α(OH)D_5, EB1089, RO24-2637, 22-oxa-calcitriol, MC903	All Effective	VDR+
	Du-145	1,25$(OH)_2D_3$, RO23-7553	Ineffective	VDR+/−
	Du-145	RO24-5531, RO26-2198	Effective	
Colon	HT-29, CaCo-2	1,25$(OH)_2D_3$, RO24-5531	Effective	VDR+

cer cells expressing positive efficacy for viamin D analogs are VDR positive. It has also been reported that the low VDR expresser PC3 and DU 145 cells poorly respond to the vitamin D as compared to LnCap cells. However, transfection of VDR cDNA was sufficient to establish growth responsiveness in PC3 and DU 145 cells. These results suggest that the presence of VDR is essential for the responsiveness of vitamin D, however the content may not directly correlate with the efficacy of the analog in prostate cells [37]. At the same time, the efficacy of vitamin D analogs did not correlate with the affinity of binding to VDR [38]. The majority of the analogs express lower affinity for VDR as compared to 1,25-dihydroxy D3, and yet they inhabited cell proliferation as effectively as the active metabolite. As with other compounds, 1α(OH)D5 also inhibited LnCap cell growth at 10−7 M concentration (unpublished). These results indicate that besides VDR, other factors may also influence action of vitamin D in cancer cells (Table 1).

2.3. Efficacy of vitamin D analogs in vivo

In order to establish possible clinical significance of vitamin D in preventing or treating cancer, it is essential to evaluate its activity in experimental models. Although over the past several years there is a considerable effort diverted towards evaluating chemopreventive effects of analogs of vitamin D in carcinogen induced experimental tumor models, very little mechanistic studies have been carried out. Unlike homogenous cell type in tissue culture, in vivo studies are much more complex and there is heterogeneity of cell types and presence of tissue interactions. Nonetheless, it is extremely important to establish the role of a chemopreventive agent in carcinogenesis models prior to understanding its mechanism(s) of action. Here, we have summarized current literature regarding the protective effects of analogs of vitamin D. The prerequisites for chemoprevention experiment are to ascertain that the agent is effective at a non-toxic concentration [29]. One of the primary side effect of vitamin D is hypercalcimia because of vitamin D treatment [39,40]. Therefore, the agent has to be active at non-hypercalcimic concentration. It is also important to mention that some analogs may be non-calcemic and yet can not be tolerated at high concentrations. Therefore, in such cases it is necessary to monitor the toxicity of the agent in a dose response study. This is usually achieved by establishing a maximum tolerated dose for each chemopreventive analog of vitamin. So far, there are only a handful of analogs evaluated in vivo for their efficacy in chemoprevention. These include RO24–5531 (Hoffman-LaRoche), EB 1089, CB 966, MC903 (Leo Pharmaceuticals), 22-oxa-calcitriol (Chugai Pharmaceuticals Japan) and 1α(OH)D5 (OncQuest Inc.). Although experimental models for carcinogenesis are available for several target organs, effects of vitamin D analogs have been studied mainly in mammary and colon carcinogenesis with sparse reports on a few other organs. The results are summarized in Table 2.

2.4. Mammary carcinogenesis

The most widely utilized models are 7,12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitrosourea (MNU). Both carcinogens induce mammary adenocarcinoma in rats with nearly 100% incidence. The time course of appearance of tumors and their response to ovarian hormones is well worked out [41]. Nearly all the tumors induced by MNU are ovarian hormone dependent whereas 80% of the tumors developed in response to DMBA are hormone dependent. The other 20% tumors induced by DMBA are fibroadenoma. The histopathological evaluations reveal very close similarities between tumors induced by these carcinogens in rats and human breast cancer pathology. These tumor models are extensively used for evaluation of chemopreventive agents for their efficacy [42]. In earlier studies it was ob-

Table 2
Summary of efficacy of vitamin D analogs in chemical carcinogenesis models

Organ	Models	Analog	Dose	Efficacy	Comments
Breast	MNU-induced adenocarcinoma	RO24-5531,	1,10 nmole/kg diet	Effective	No toxicity
		1α-Hydroxyvitamin D_5	58.4, 116.8 nmole/kg	Effective	No hypercalcemia
				Dose related effect	No loss of body weight
		1α-hydroxy D_3	0.25 nmole	growth inhibition	Treatment schedule
		$1,25(OH)_2D_3$	0.59-2.99 nmole/kg	No Effect	Hypercalcemia
		MC903	111 nmole/kg	Growth inhibition	Hypercalcemia
		EB1089	1.1-5.5 nmole/kg	Effective	Hypercalcemia
					Loss of body weight
Prostate	MNU-induced	RO24-5531	10 nmole/kg	Effective	No toxicity
					No effect on dorsal prostate
		$1\alpha(OH)D_5$	58.4-116.8 nmol/kg diet	In progress	
Colon	AOM-induced	RO24-5531	2.5 nmole/kg ip	Effective	No toxicity
	DMH-induced	22-oxa-Calcitriol	72.5 nmole/kg ip	Effective	
	DMH-induced	24R,25 dihydroxyvitamin D_3	0-24 nmole/kg	Effective	Reduced aberrant crypt
	DMH, MNU, and nitrosamines	24R,25 dihydroxyvitamin D_3	0-12 nmole/kg	Effective	foci colon only

served that treatment with 1,25 dihydroxyvitamin D3 up to 3 nmole/kg BW of rat resulted in no protection against mammary carcinogenesis and yet increased calcium levels in blood was reported [43,44]. Two other vitamin D analogs studied in this report included EB1089 and MC 903. MC903 at a very high dose provided some prtection against mammary tumor growth whereas EB1089 was effective at all the doses evaluated [45,46]. However, there was hypercalcimia observed at <2 nmole (1 and 2.5 μg)/kg dose level. An in depth study to evaluate effects of R024–5531 against MNU-induced mammary carcinogenesis has also been reported. Anzano et al showed that this non-calcemic analog was effective against both the incidence and multiplicity of mammary tumor development at very low levels of 2.5 nmole per kg of diet [24]. However, this effect was observed only when low carcinogen dose was employed. At higher carcinogen dose, level there was no effect against the tumor incidence. Higher than 2.5 nmole per kg of diet dose level was not evaluated in this study, it is possible that it induces toxicity other than hypercalcimia at higher concentrations and 2.5 nmole may in fact be maximum tolerated dose in rats. In a more recent study, we evaluated effects of 1α-hydroxyvitamin D5 in MNU-induced mammary tumor model. The results showed that the animals could tolerate 116 nmole/kg (50 μg/kg) diet concentration of the analog during a six-week toxicity study without adversely affecting serum calcium levels. In older animals, dietary treatment with 0.116 μmole/kg diet 1α-hydroxyvitamin D5 reduced both the incidence and multiplicity of MNU-induced mammary tumors [47]. In this experiment, the vitamin D supplementation began two weeks prior to the carcinogen treatment and continued through out the experiment (Table 2). This meant that both initiation and promotion phases were not separated and the dietary modulation was included during both phases. The selectivity between these two stages in relation to 1α-hydroxyvitamin D5 effect is currently in progress.

2.5. Colon carcinogenesis

There are several well-established colon carcinogenesis models available for evaluating effects of chemopreventive agents. Carcinogens successfully utilized for induction of colon cancers are MNU, 1,2, dimethylhydrazine and azoxymethane. The time frame of induction of aberrant crypts and carcinomas of colon by DMH and AOM have been established [48]. Analogs 1α-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 have been used against DMH induced colon carcinogenesis. Rats received 20 weekly injections of 20 mg/kg DMH. 1,25, Dihydroxyvitamin D3 at a concentration of <0.3 nmole reduced the incidence of colon carcinomas from 46% to 11%. However, there was hypercalcimia associated with this efficacy. In a separate study, effect of R024–5531 was also studied in colon carcinogenesis. Dietary inclusion of R024–5531 for 34 weeks resulted in 40% reduction of colon cancer formation in treatment groups [49]. None of the tumors developed in vitamin D treated rats was adenocarcinoma, they were all benign. Thus, RO24–5531 appears to be very effective against colon cancers. In another study, Otoshi showed that the IP injections of 22-oxa-cxalcitriol also suppressed the development of aberrant crypt foci in rats [50]. The analog, 1α-hydroxyvitamin D5 has not been evaluated for its efficacy in colon carcinogenesis (Table 2).

2.6. Transplantable models

Unlike chemically induced carcinogenesis models, transplantable models are used to evaluate effects of test agents on the growth of the established tumor cells. Since these studies are largely conducted with human cancer cells growing in culture, athymic mice are used as animal of choice. Surprisingly, not all cancer cells form tumors in athymic mice Earlier we had reported that breast cancer cells mixed with matrigel in the ratio of 1:1 results in a remarkable

increase in the development of tumors. Since our original report [51], the use of matrigel for better response in athymic mice has become a common practice for breast cancer. On the other hand, melanoma, sarcoma, colon cancers and prostate cancers typically are not mixed with matrigel to grow in nude mice. Effects of 1,25 dihydroxyvitamin D3 and synthetic analogs of vitamin D3 have been evaluated for their anticancer efficacy on the growth of many cancer types. Studies from our laboratory have shown that 1α-hydroxyvitamin D5 inhibited growth of steroid receptor positive MCF-7 as well as ZR75A cells in vivo [27]. Both these cell lines are positive for both ER and VDR. Cell line established in our laboratory, BCA-4, which is positive for VDR but negative for estrogen and progesterone receptors also responded to the D5 analog of vitamin D. The responsiveness was observed at 0.3nmole i.p. injections or by dietary incorporation of 30 nmole D5-analog/kg diet. These results suggested that the presence of VDR was essential for the efficacy of vitamin D analogs and steroid receptors were of less significance. This was further confirmed by demonstrating lack of effect of 1α (OH) D5 in MDA-MB231 cells that either lack VDR or are relatively very low in expression of VDR expression [27,52].

Effects of 1,25-dihydroxyvitamin D3 was evaluated and compared with EB1089 in transplantable prostate tumor model using androgen-insensitive metastatic rat prostate model. MAT LyLu cells were injected in Copenhagen rats and appropriate groups were treated with low (0.5 μg/kg) and high (1 μg/kg) doses. Both these analogs reduced the metastatic foci in lungs in these rats, however the effect was accompanied by hypercalcemia and loss of body weight at higher dose [53]. More recently, we evaluated effects of 1α(OH) D5 on the growth of LnCap cells in athymic mice (unpublished). Results showed that 55 nmole/kg (25 μg/kg) of diet of the vitamin D analog for 60 days resulted in reduced tumor volume as compared to the control LnCap tumors. At 55 nmole/kg diet concentration, the D5 analog did not elevate concentration of serum calcium levels. Thus, the experimental evidence indicates that not only vitamin D analogs are effective as chemopreventive agents in experimental carcinogenesis models but they also suppress the growth of human cancer cells in athymic mice. Not many studies have been reported to establish the role of vitamin D analogs in preventing or retarding the metastasis of cancer cells to a distant organ, however a couple of reports as described above clearly hint that the selective analogs may be very influential against the cancer cell metastasis. Again, mechanistic studies have not been carried out in these models.

3. Clinical application of laboratory research

As described above vitamin D and its analogs have been examined for their efficacy in numerous in vitro and in vivo models to identify the most potent and yet non-toxic chemical forms of vitamin D. Many of these synthetic analogs have been evaluated in one or multiple models [16]. The reason for lack of analogs, which qualify for further evaluations, possibly in the clinical trials and subsequently as a chemopreventive or chemotherapeutic agent, is the toxicity to efficacy relationship. If a compound were toxic at a concentration which is effective in preventing experimental carcinogenesis or in suppressing cancer growth in experimental models then that analog would be of little value. This is one of the major reasons why 1α,25-(OH)2 D3, the active metabolite of vitamin D has not been employed in cancer prevention or treatment schedules. As discussed previously, the concentration, at which 1α,25 dihydroxy vitamin D3 is efficacious, is also sufficient to induce hypercalcemia in experimental animals. This was first observed by Koeffler and colleagues [54], by evaluating induction of cell differentiation of blast cells taken from the patients with acute myelogenous leukemia. Concentrations of 1μM induced cell differentiation but also was found to be toxic. The studies were extended by treating patients with myelodysplastc syndrome with 2 μg/day of 1,25(OH)2D3. Results also showed that 9 out of 18 patients developed hypercalcimia. In another study, safety and efficacy of both oral and topical treatments of 1α,25(OH)2D3 were evaluated for psoriasis. A study with 85 patients who received calcitriol (1α,25 (OH)2D3) showed that 88% of the patients showed some improvement in the disease. Among those responded to treatment, 26% showed complete protection from psoriasis. There was a significant increase in the calcium excretion, however renal function remained unaffected [55]. A similar mall clinical study was also carried out with 84 patients. The majority of the patients in this study responded to the topical treatment of 1.5 μg of calcitriol. No calcium metabolism abnormalities were observed. The study concluded that topical calcitriol was safe and effective for the treatment of psoriasis [56]. These early reports led to development of relatively nontoxic analogs of vitamin D. The agents that have received considerable attention include EB1089 (seocalcitol), MC903, RO24–5531, 1α-hydroxyvitamin D2, 25-hydroxyvitamin D3, 19-nor-1α,25-dihdyroxyvitamin D2 and 1α-hydroxyvitamin D5 [57,58,59]. These agents have been considered acceptable, based on the preclinical toxicity in animals under stringent experimental conditions. Early results with EB1089 confirmed low calcemic activity in a human maximum tolerated dose finding Phase I/II study [55]. Similar Phase I clinical trial with 36 patients with advanced breast cancer or colorectal cancers is also completed. The maximum tolerated dose of 16–24 nmole/m2 (total daily dose of 20–40nmole) for EB1089 was reported as compared to 2–4 nmole for 1,25-dihydroxyvitamin D3 [60]. Several reports in Japanese have also appeared for toxicity in experimental animals for MC903. Based on this clinical trials have been conducted for psoriasis using this analog, however the clinical trials for cancer patients have not been reported.

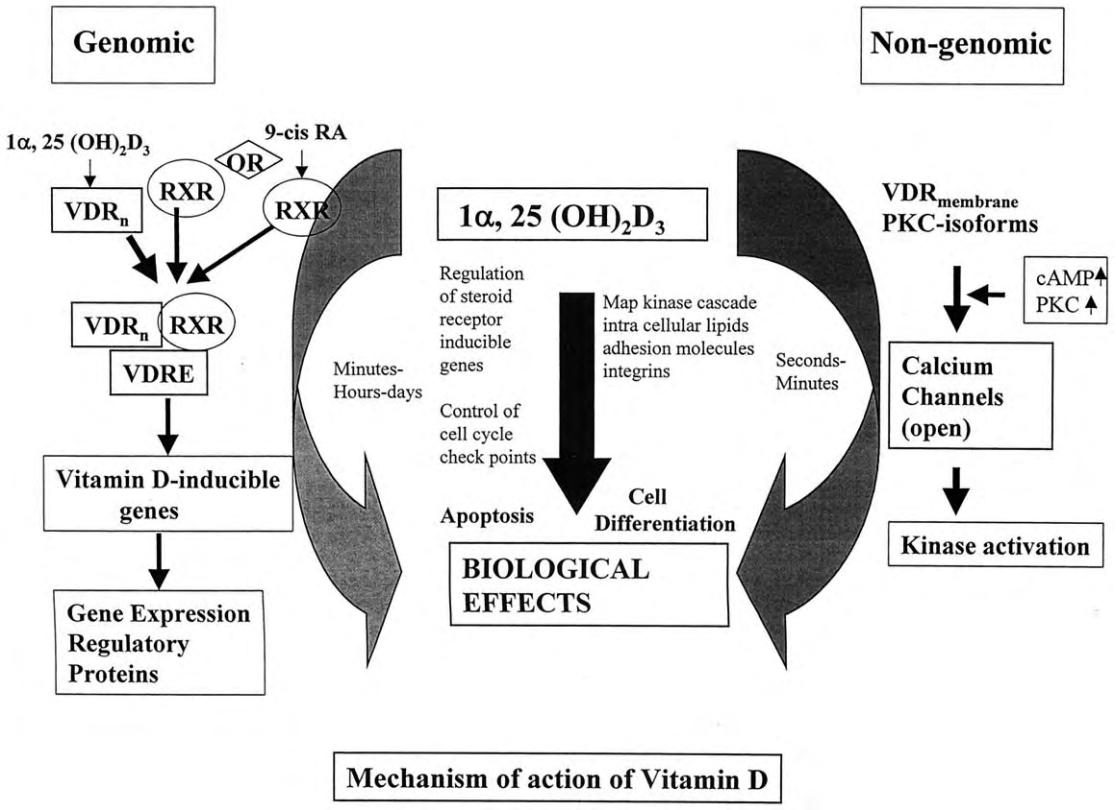

Fig. 4. Schematic diagram of potential mechanism of action of vitamin D. Vitamin D functions both via genomic and non-genomic pathways. Possible pathways of both these mediations of vitamin D action are shown in this diagram.

4. Mechanism of action of vitamin D and cancer

Vitamin D is classified as a steroid hormone [16]. The most unique feature for the steroid hormone has been its association with the specific nuclear receptors. The functional significance of the receptor-associated ligand is the initiation of a cascade of events involving signal transduction eventually leading to the biological function. As shown in Fig. 4, there are two distinct modes of action for vitamin D, one mediating vitamin D action via its binding with high affinity to its specific protein receptor (vitamin D receptor, VDR) and the second involving rapid functions using non-genomic membrane associated functions [61,62]. The non-genomic actions are generally very rapid, often the response could be within minutes as compared to genomic actions which may take longer period for the response. Vitamin D is unique in this respect since both these pathways have been well worked out and are supported by extensive evidence.

4.1. Genomic actions of vitamin D

Consistent with all other steroid hormones vitamin D mediates its action via VDR. Identification of VDR was initially made in chicken intestines by Haussler and Norman [63], followed by its preferential uptake by mammalian intestines and cell free binding of the cytosol to radioactive 1,25(OH)2D3, resulting in the saturable binding with a dissociation constant of 10−9 M. The sucrose density gradient studies of cytoplasmic VDR showed sedimentation of 3.5 S. Subsequently it was observed that the active metabolite associated with cytoplasmic VDR could bind to chromatin fractions [64]. Results have been reported over the years indicating localization of VDR in a variety of target organs and tissue types. These include digestive tract (esophagus and colon), mammary glands, prostate glands, lung alveolar cells brain neurons, connective tissues, fibroblasts, testes and ovaries as well as bone and osteoclasts. These results formed the basis for the establishment and future studies on VDR and its functional significance.

The VDR mRNA in human is a 4.8 kb whereas VDR is a 60 kD protein ranging from 400 to 27000 copies per cell yielding 10 to 100 femto (10−15)-moles/mg protein. Using anti-VDR antibody 9A7 cDNA, libraries derived from chicken intestine were screened in a viral expression system. Protein generated from a single clone from this screen reacted with the anti-VDR antibodies. Since then, VDR-cDNA has been sequenced using monoclonal antibody selection process [6]. Numerous reports collectively have concluded that there is a cluster of hormone receptors forming a family of steroid hormone receptor gene family [66]. Common structural motifs containing DNA binding do-

mains associated with regulatory domains are conserved during the evolution. These genes are under a direct control of transcription factors which regulate biological functions of cell proliferation, differentiation and death. As it is well established steroid hormone receptors are divided into five sections termed A through F. The segments A/B includes residues amino terminal to DNA binding domain, whereas C region contains highly conserved DNA binding domain. The ligand-binding domain at the carboxyl end is termed as either E or E/F region. The hinge on the other hand between C and E segments is termed as D region. While C region of the DNA binding domain is highly conserved, E section is the most flexible region. All regulatory controls reside in this region [67]. Within the ligand binding domain there are both homologies and structural differences among steroid hormones, which make them significantly different from other nuclear receptors including estrogen receptor, retinoic acid receptors, progesterone receptors, peroxisome proliferator activated receptor and thyroid hormone receptors [68]. In order for VDR to function, it needs to interact with vitamin D response element (VDRE) and bind to DNA. VDRE is a two identical hexanucleotide sequences separated by a spacer of 3 nucleotides. The spacer sequence is not conserved. Unlike estrogen receptors, this repeated sequence of two six-nucleotide segments, suggest that VDR must form a dimer for its action. Recent experiments have shown that VDR heterodimerizes with nuclear accessory factor (NAF) or retinoid X receptors (RXR). The natural metabolite 1,25(OH)2D3 transactivates VDRE in VDR positive cells but fails to show interaction in CV-11 (VDR-) cells. These results imply that the synthetic analogs transactivating VDR-VDRE interaction probably mediate their function via genomic pathway in a manner similar to dihydroxyvitamin D3 [69]. Results generated from our laboratory have shown that CV-1 cells transfected with VDR and VDRE when incubated with 1α(OH)D5 showed enhanced transactivation of VDR. Similarly, T47D and ZR75 ER+ VDR+ breast cancer cells express basal level of interaction with transient transfection of VDRE. However, co-transfection of VDR and VDRE significantly enhance the VDR-VDRE interaction when the cells are incubated with 1α(OH)D5 [29,70]. These results indicate that this analog of vitamin D mediates its action via genomic pathway.

Interactions among VDR and other receptors within the steroid receptor family have been a subject of a few investigations in recent years. Since the estrogen receptor positive and negative cells respond differently to vitamin D, in recent studies effects of 1,25-dihydroxyvitamin D3 on ER regulation have been investigated [71]. Results showed that all D-analogs evaluated, EB1089, KH-1060, R023–7553 down regulated ER levels when measured by western blot analysis as well as ligand binding assays. Moreover, this reduction was correlated with steady state levels of ER mRNA indicating direct down regulation of ER transcription by vitamin D analogs. In these studies, induction of progesterone receptors by estrogen was also reduced. More recently, in our laboratory we determined role of 1α-hydroxyvitamin D5 on cell cycle arrest and expression of progesterone receptors in BT474 cells. Results showed that cells were arrested in G1 phase accompanied with apoptosis down regulated estrogen inducible progesterone receptors [72]. Similar studies have also been conducted in prostate cancer cells to determine if vitamin D interacts with androgen receptors. Similar to ER+ breast cancer cells, androgen receptor (AR) positive LnCap cells respond better to vitamin D analogs compared to androgen resistant cells. Human glandular kallikerin (hK2) is an androgen regulated protein expressed in LnCap cells. Recent studies provide evidence for the role of vitamin D analogs for signaling pathways for androgen receptors [73].

The action of steroid hormone is regulated by various factors such as the receptor subtypes, regulation of hormone responsive gene promoters and the activation or suppression of function in response to steroid receptor complex. .Several cellular signaling pathways are involved in the regulation of gene expression by the steroid hormone receptors. The transcriptional activity of some hormone receptors is enhanced by protein kinase activators and growth factors. These proteins stimulate steroid receptor phosphorylation. These findings suggest that changes in steroid receptor phosphorylation are important in determining biological effects of these hormones and their receptors [74]. Alternatively, estrogen receptors could be activated by signals from tyrosine kinase-linked cell surface receptors. This process also involves phosphorylation of the kinases or the transcription factors [75]. Thus, either receptor phosphorylation or ligand bound receptor mediated phosphorylation of other factors is important for the receptor function. VDR like other receptors also get phosphorylated on the serine residues. The extent of phosphorylation is correlated to the extent of responsiveness of the cells to 1,25(OH)2D3 or calcitriol [76]. Furthermore, the phosphorylation is also correlated with VDR-VDRE interaction in transiently transfected cell system. These results suggest that 1,25(OH)2D3 mediated transcription may be dependent on VDR phosphorylation. Phosphorylation of human VDR has also been reported, however the extent of hVDR phosphorylation is significantly lower than the rat VDR phosphorylation [67]. In human, the majority of VDR phosphorylation is located at Serine 51. It has been fairly well established that the ser-51 phosphorylation is regulated by protein kinase C. The phosphorylatable residue at ser 51 is also observed for retinoic acid, thyroxin, and estrogen receptors. However, PKC-mediated phosphorylation is unique to VDR, the functional significance of PKC mediated phosphorylation is not conclusively demonstrated [77]. Both genomic activation by PKC-mediated phosphorylation and inhibition of VDR binding to DNA by this phosphorylation process has been reported [78]. In the later case, it is proposed that PKC dependent phosphorylation create a negative feed back loop that reduces availability of VDR for DNA binding. In addition to VDR phosphorylation by PKC, casein kinase II

and protein kinase A have also has been shown to phosphorylate VDR. Based on the working model for vitamin D action as proposed by Mark Hausler and colleagues [67], it is assumed that VDR resides in target cell nucleus are associated with DNA in a monomeric weak inactive confirmation. Upon binding with the ligand, VDR may become phosphorylated. Moreover, this complex allows dimerization of VDR with RXR. Phosphorylation of VDR and its heterodimerization allows inactivation of the repressor molecules. Haussler proposes that VDR-vitamin D complex dimerization with unliganded RXR makes it unresponsive to 9-cis retinoic acid. On the other hand, if RXR is preoccupied with its ligand then it can form homodimers as well as heterodimers. The homodimers may then disallow the vitamin D interaction with VDR and VDRE interaction. Needless to say that the understanding of genomic regulation of VDR mediated vitamin D function is far from complete. Yet, tremendous progress has been made to elucidate a delicate balance between receptors their interactions, phosphorylation of receptors and their regulatory proteins in order to understand molecular genomic mechanisms of vitamin D action.

4.2. Nongenomic rapid actions of vitamin D

While there is a wealth of information available and is constantly getting updated on the genomic actions of steroid receptors, not all actions of vitamin D can be explained by the genomic regulations. Anthony Norman and his colleagues have been studying the non-genomic action of vitamin D for the past 20+ years and have elegantly demonstrated that in addition to genomic actions of vitamin D there are rapid actions of the hormone largely mediated by membrane receptors of vitamin D and PKC [79,80]. The early studies demonstrated vitamin D mediated stimulation of calcium transport in chick duodenum called transcaltachia. Typically, the vitamin D induced initiation of responses in transcaltachia is not mediated by nuclear VDR directed signal transduction pathways. These responses occur within minutes unlike the genomic expression, which may take days prior to the modulation of endpoint markers. The rapid responses involve membrane receptors of vitamin D, and the pathways involved in induction of calcium channels leading to the exocytosis of calcium bearing vesicles from lysosomes. The ligand binding domain of the plasma binding protein, nuclear VDR and membrane VDR require unique shape of confirmationally flexible 1α,25(OH)2D3. The orientation and rigidity of the flexible side chain as well as the position of A ring in relation to C/D rings determine the vitamin D action. For example, the non-genomic responses including opening of the chloride channels, activation of PKC and MAP kinases require a planar 6-s-cis ligand shape which is recognized by the membrane-VDR as opposed to 6-s-trans bowl shaped 1α, 25(OH)2D3 required for nuclear-VDR interactions [81,82]. Involvement of nongenomic pathways for vitamin D action in carcinogenesis or prevention and therapy of cancer is not clearly defined, however in recent years increasing evidence for rapid effects of steroids that are incompatible with the classical genomic actions is accumulating. Norman and colleagues presented a Mannheim classification of nongenomic action at the first International meeting on the rapid actions of steroid hormones in Mannheim, Germany in 1998 [83]. According to this definition, the nongenomic action of steroid hormones is divided into six categories arbitrarily termed as A1, AIIa, AIIb, BI, BIIa, and BIIb. These differences in various types were according to the functional properties of the hormone. Type AI was classified as nongenomic direct action of steroids at high concentrations that does not require hormones. AIIa is direct action requiring classical receptor for example ERα induction of nitric oxide synthatase. Classification AII-b relates to a nongenomic rapid response transmitted by membrane receptors. Steroid hormones such as estradiol, mineralocorticoids, and vitamin D do function via membrane receptors. Finally, BII-b is the action of steroid hormone where the steroid functions as an agonist. There are several examples of such action in neuroendocrinological, function. However this would be out of scope for the current review and is not described in detail. The functional significance of BI and BII-a is not defined. Despite these developments, the rapid responses of vitamin D are not understood well in relation to the vitamin D action in cancer prevention or therapy.

In summary, in recent years role of synthetic analogs in the management of cancer patients has been extensively evaluated. Several hundred analogs of vitamin D have been synthesized and evaluated for their toxicity and efficacy in a variety of experimental models. To date only a few analogs have been considered for further development. Although it is generally accepted that the action of vitamin D is mediated via both genomic and non-genomic pathways, the major emphasis for the antiproliferative action of vitamin D analogs is placed on the VDR mediated action of the hormone. The VDR- breast and prostate cancer cells do not respond to vitamin D analogs. In steroid hormone receptor positive breast and prostate cancer cells the vitamin D acts by regulating steroid hormone inducible genes, whereas in the steroid receptor negative cells vitamin D induces cell differentiation. Understanding of the molecular mechanism of action of vitamin D will be crucial in generating more efficacious analogs of vitamin D in the prevention and treatment of cancer.

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Efficacy and Mechanism of Action of 1α-hydroxy-24-ethyl-Cholecalciferol (1α[OH]D5) in Breast Cancer Prevention and Therapy

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Abstract

It is now well established that the active metabolite of vitamin D_3, 1α,25$(OH)_2D_3$, regulates cell growth and differentiation in various in vitro cancer models. However, its clinical use is precluded due to its hypercalcemic activity in vivo. Hence, several less calcemic vitamin D analogs have been synthesized and evaluated for their chemopreventive and therapeutic efficacy in experimental carcinogenesis models. A novel analog of vitamin D_3, 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5), has currently been under investigation in our laboratory for its application in breast cancer prevention and therapy. 1α(OH)D5 had been shown to inhibit development of estrogen- and progesterone-dependent ductal lesions as well as steroid hormone-independent alveolar lesions in a mammary gland organ culture (MMOC) model. Moreover, the inhibitory effect was more significant if 1α(OH)D5 was present during the promotional phase of the lesion development. The growth inhibitory effect of 1α(OH)D5 has also been manifested in several breast cancer cell lines, including BT-474 and MCF-7. Breast cancer cell lines that responded to 1α(OH)D5 treatment were vitamin D receptor positive (VDR+). Vitamin D receptor-negative (VDR−) cell lines, such as MDA-MB-231 and MDA-MB-435, did not show growth inhibition upon incubation with 1α(OH)D5. This suggests the requirement of VDR in 1α(OH)D5-mediated growth effects. Interestingly, breast cancer cells that were VDR+ as well as estrogen receptor positive (ER+) showed cell cycle arrest and apoptosis, while VDR+ but ER− cells (UISO-BCA-4 breast cancer cells) showed enhanced expression of various differentiation markers with 1α(OH)D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly down-regulated in ER+ BT-474 cells with 1α(OH)D5 treatment. This implies a differential effect of 1α(OH)D5 on ER+ vs. ER− cells. Additionally, comparison between the effects of 1α(OH)D5 on normal vs. transformed cells indicated that 1α(OH)D5 does not suppress cell prolifera-

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tion of normal epithelial cells but selectively targets growth of transformed cells. We extended our experiments to determine in vivo effects of 1α(OH)D5 using an MNU-induced mammary carcinogenesis model in female Sprague-Dawley rats. Results showed that 1α(OH)D5 (25–50 μg/kg diet) decreased the incidence and multiplicity of mammary tumors in these rats. In addition, it increased the latency period of early precancerous lesions. Similar studies, with DMBA as a carcinogen in younger rats, showed that 1α(OH)D5 supplementation was effective in reducing onset of carcinogenesis in rats and the effect was largely reflected during the promotional phase of carcinogenesis. Recently, a preclinical toxicity profile for 1α(OH)D5 was completed in rats and dogs that provides estimation of the maximum tolerated dose in mammals. Based on our findings, we will shortly be initiating a 1α(OH)D5 phase I clinical trial for breast cancer patients.

Introduction

Breast cancer is generally characterized by transformation of normal to atypical hyperplastic epithelium, with subsequent risk of progression to intraductal carcinoma and in some cases invasion into stroma (Mallon et al. 2000). Breast cancer is the second leading cause of cancer-related deaths among women in the US, with about 180,000 new cases and 46,000 deaths annually (Edwards et al. 2002). Although the overall incidence of breast cancer has not been reduced in the last decade, the breast cancer-related mortality has been decreasing with approximately 3.4% annual decrease from 1995 through 1998 in the US (Howe et al. 2001; Peto et al. 2000). This decrease in mortality is probably a result of availability of greater screening efficiency and better chemopreventive and therapeutic strategies. Despite increased survival rates, breast cancer results in considerable morbidity and patient care costs. Chemoprevention is an important aspect of curbing the progression or recurrence of the disease. The chemopreventive agents usually include natural or synthetic compounds that can either prevent transformation or inhibit proliferation of transformed cells by inducing apoptosis, growth arrest or differentiation of initiated and transformed cells (Rosenbaum-Smith and Osborne 2000). Several classes of compounds have been under investigation in this regard. These include selective estrogen receptor modulators, retinoids, deltanoids (vitamin D derivatives), phytoestrogens, flavonoids, and aromatase inhibitors, among others (Kelloff et al. 1996).

On a global basis, breast cancer incidence is fivefold higher among middle-aged women in the Western countries than in women from Asian countries. Various diet and lifestyle as well as genetic factors have been implicated in the high occurrence of breast cancer in the Western world. Some epidemiological studies have shown association of lower sunlight exposure to higher breast, colon, and prostate cancer mortality rates in the US and other Western countries (Freedman et al. 2002; Polek and Weigel 2002; Garland et al. 1990; Gorham et al. 1990). This is consistent with reports of an association of breast

cancer mortality with lower serum vitamin D_3 levels (John et al. 1999; Christakos 1994). Lower serum vitamin D_3 levels could be due to lower sunlight exposure as well as lower dietary intake.

The biologically active metabolite of vitamin D, 1α,25$(OH)_2D_3$ or calcitriol, is a steroid hormone that was identified in the early 1920s as an antirachitic substance (Carpenter and Zhao 1999). Later it was established that vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol by the action of ultraviolet radiation. Vitamin D_3 is activated subsequently in liver and kidney by the hydroxylation reactions at C25 and 1α positions to yield 1α,25$(OH)_2D_3$. Calcitriol has been known to exert calciotrophic effects, mainly through increasing calcium uptake in the intestine for regulation of bone health. Aside from its role in calcium homeostasis, vitamin D_3 is involved in regulation of various cellular processes. Vitamin D_3 binds to nuclear vitamin D receptor (VDR) and undergoes conformational changes, which allow VDR to function as a transcription factor (Jones et al. 1998; Haussler 1986). Earlier, VDR was found to be present in abundance in intestine, bone, liver, and kidney cells. Aside from the classic target organs, VDR has now been isolated from a variety of tissues, including normal mammary epithelium as well as breast tumors (Friedrich et al. 1998; Buras et al. 1994; Eisman et al. 1980).

In order for VDR to function, it needs to interact with vitamin D response elements (VDRE) and bind to DNA to initiate or repress transcription (Pike 1991). VDR must form a dimer to stabilize VDRE transactivation (Jones et al. 1998). Most common partners for VDR heterodimerization are nuclear accessory factor (NAF) and retinoid X receptor (RXR) (Rachez and Freedman 2000). VDR transactivation of VDRE results in regulation of a wide variety of genes, some of which are involved in cell growth and proliferation. Vitamin D_3 also exerts some nongenomic rapid responses possibly through a putative membrane receptor (Falkenstein et al. 2000).

The presence of VDR in the normal mammary epithelial cells suggests a role of calcitriol in the regulation of mammary gland function. The levels of VDR in mammary tissue increase during pregnancy and lactation and decrease as the glands regress back to normal size (Zinser et al. 2002; Narvaez et al. 2001). VDR knockout mice have been shown to have larger mammary glands than normal mice; it has also been shown that the glands would not regress back to prepregnancy size at the termination of lactation (Zinser et al. 2002). This suggests that vitamin D mediated signaling may be very important for maintaining the normal cycling of the mammary gland. Various case studies indicate that a high percentage (60%–80%) of breast cancer epithelia contain VDR (Friedrich et al. 1998) and that there is a positive correlation between VDR polymorphisms and increased risk of breast cancer (Bretherton-Watt et al. 2001; Lundin et al. 1999). These reports further signify vitamin D_3-mediated signaling to be of importance in regulating healthy mammary gland. In cell culture models, vitamin D_3 has been demonstrated as an inducer of growth arrest and differentiation in various cancer cell lines, including breast cancer cells (Hisatake et al. 2001; Welsh et al. 1998; James et al. 1997). Taken together, these results warrant potential use of vitamin D_3 in cancer preven

tion and therapy. However, due to its hypercalcemic activity, vitamin D_3 can not be administered at doses that would be effective for chemoprevention or therapy. Adverse effects of vitamin D_3 at cancer-preventive doses are hypercalcemia, soft tissue calcification, weight loss, and possibly death (Roder and Stair 1999; Vieth, 1999).

Since the early 1980s, there has been a search for a vitamin D_3 analog that would selectively modulate VDR to produce growth-regulating effects without interfering with the calcium metabolism. Several analogs have been synthesized and tested for this purpose; but only a few have shown promising results in cell culture and animal models. Vitamin D_3 analogs currently being evaluated for breast cancer prevention include seocalcitol (EB-1089), calcipotriol (KH-1060), Maxacalcitol (OCT), RO-24–5531, and 1α(OH)D5 (Mehta and Mehta 2002; Guyton et al. 2001). In this review, we summarize the results from experiments conducted in our laboratory that elucidate the potential role of 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5) in breast cancer prevention or therapy.

Synthesis and Characterization of Vitamin D Analog, 1α(OH)D5

As mentioned earlier, vitamin D_3 can be obtained from food as well as synthesized in the skin through the action of sunlight. Vitamin D_3 belongs to the family of 9,10-secosteroids which differ only in side-chain structure (Napoli et al. 1979). Other forms of D-compounds include D2, D4, D5, and D6. In the late 1970s, major interest in the synthesis of these compounds was to evaluate them for use in management of renal osteodystrophy and osteoporosis. In this regard the calcemic activity of D series of compounds was compared and D5 was found to be the least calcemic of all (Napoli et al. 1979), a property that would later prove useful in its possible application for cancer prevention. The D5 form is also known as irridated 7-dehydrositosterol. The hydroxylated form of D5 (1α[OH]D5) was synthesized as described previously (Mehta et al. 1997a).

Briefly, β-sitosterol acetate was converted to 7-dehydro-β-sitosterol acetate by allelic bromination and dehydrobromination. Lithium aluminum hydride and tetrahydrofurane were used to reduce 7-dehydro-β-sitosterol to 7-dehydro-3β-sitosterol. The reaction mix was sequentially subjected to photolysis and thermolysis to yield 24-ethyl-cholecalciferol (D5). D5 was hydroxylated by Paaren-DeLuca hydroxylation sequence to produce 1α(OH)D5. The product was crystallized and characterized by ^{1}H nuclear magnetic resonance at 400 Hz and mass spectroscopy. The purity was assessed by high-pressure liquid chromatography. The following properties were observed: melting point, 150–152°C; UV λ-max, 265 nm; molar extinction coefficient (ε), 18000; molecular weight, 428.7. The major structural differences between biologically active vitamin D_3 and 1α(OH)D5 are the lack of hydroxylation at the C-25 position and the presence of an ethyl group at the C-24 position in the 1α(OH)D5 molecule (Fig. 1).

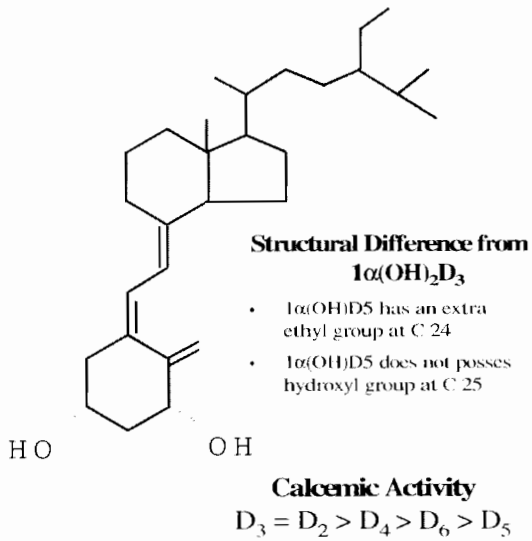

Fig. 1 Structure of 1α(OH)D5 and its Ca^{++} mobilizing activity in mammals in relation to other primary vitamin D series compounds

Calcemic Activity of 1α(OH)D5

Earlier studies in DeLuca's lab had shown that among the known vitamin D series of compounds (vitamin D_2–D_6), D5 is the least calcemic of all (Napoli et al. 1979). D5 was found to be 80-fold less active than vitamin D_3 in the intestine and about 100- to 200-fold less active in bone in mobilizing the Ca^{++} stores (Napoli et al. 1979). The calcemic activity of the hydroxylated form was not known. Therefore, we measured calcemic activity as well as body weight change in animal models to determine the maximum tolerable dose and toxicity of 1α(OH)D5. In the first experiment, 3-week-old Sprague-Dawley male rats were fed a vitamin D_3-free diet containing 0.47 g calcium and 0.3 g phosphorus/100 g diet (Mehta et al. 1997a). These rats were kept under yellow light to create a vitamin D_3-deficiency state. After the rats were fed a vitamin D_3-deficient diet for 3 weeks, their plasma calcium levels were measured and rats with calcium levels under 6.0 mg/dl were considered vitamin D_3 deficient. Vitamin D_3-deficient rats were administered 1α(OH)D5 intragastrically for 14 days and the plasma calcium levels were measured. The control group showed a plasma calcium concentration of 5.4+0.3 mg/dl, while the rats receiving 1α(OH)D5 at a dose of 0.042 μg/kg per day had plasma calcium concentration of 6.0+0.63 mg/dl, which was not significantly different from the control rats (Mehta et al. 1997a). On the other hand, vitamin D_3 increased

Table 1 Calcemic activity of 1α(OH)D5 in Sprague-Dawley rats

Treatment	Sample size	Dose	Plasma Ca^{++} (mg/dl)
Vitamin D-deficient male rats		(μg/kg body weight)	
Control	8	0.0	5.4±0.28
1α(OH)D5	8	0.042	6.0±0.63
1α(OH)$_2$D$_3$	8	0.042	8.1±1.2*
Vitamin D-sufficient female rats		(μg/kg diet)	
Control	15	0.0	7.0±1.19
1α(OH)D5	15	25.0	7.4±1.10
1α,25(OH)$_2$D$_3$	15	12.8	8.5±1.17*

* Significantly different from control (p <0.05)

plasma calcium concentration 50% over that of the control group (Table 1). During these experiments, the 1α(OH)D5 group did not differ in total body weight from control group. No other signs of toxicity were observed in 1α(OH)D5-fed rats compared to controls.

In a separate experiment, female Sprague-Dawley rats were fed a diet supplemented with 1α(OH)D5 to determine its calcemic activity in vitamin D_3-sufficient rats. Food was provided ad libitum. There was no body weight change at 50 μg 1α(OH)D5/kg diet in vitamin D_3-sufficient rats, while a dose of 12.8 μg 1α,25(OH)$_2$D3/kg diet was sufficient to bring about significant weight loss in the animals (Table 1). Maximum tolerated dose was determined to be 50 μg/kg diet, based on the weight and calcemic activity of 1α(OH)D5 in these rats (Mehta et al. 2000a). In addition to these experiments, we also conducted toxicity studies under the GLP using rats and dogs. For rats, the dose at which signs of toxicity first appeared was 10 μg/kg body weight (equivalent to 100 μg 1α(OH)D5/kg diet for a 150-g rat), which is twice the amount needed to bring about effective chemoprevention. However, the dogs had much lower tolerance for 1α(OH)D5 compared to rats. Based on these results, we are now conducting further studies to determine the appropriate and safe dose of 1α(OH)D5 for use in clinical settings.

Since vitamin D_3 exerts most of its effects through binding to VDR, we evaluated the ability of 1α(OH)D5 to bind to VDR. The binding affinity of 1α(OH)D5 to VDR was determined using competitive binding assays (unpublished data). Results showed that the binding affinity of 1α(OH)D5, in competition with radioactive 1α,25(OH)$_2$D$_3$ to purified VDR ligand-binding domain is 1000-fold less than 1α,25(OH)$_2$D$_3$ (Fig. 2). The IC_{50} for 1α(OH)D5 was 100 pM, while for 1α,25(OH)$_2$D$_3$, it was 0.08 pM. The lower binding affinity may explain the decreased calcemic activity of 1α(OH)D5. However, due to its lower calcemic activity, 1α(OH)D5 can be administered at much higher doses than 1α,25(OH)$_2$D$_3$. This quality can allow use of 1α(OH)D5 for prevention in the general population as well as high-risk groups. It is also important to note that the in vivo VDR affinity to its ligand is tissue specific (Napoli et al. 1979), which could not be manifested in our experiments that were conducted using

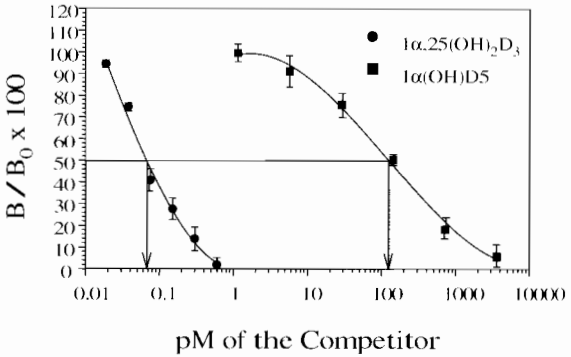

Fig. 2 Binding affinity of 1α(OH)D5 to VDR in comparison with 1α,25(OH)$_2$D$_3$

purified VDR. We have not yet critically evaluated metabolism and pharmacokinetics of 1α(OH)D5 in target organs.

Anticarcinogenic Effects of 1α(OH)D5 in In Vitro Models

The effectiveness of a variety of chemopreventive agents has been evaluated by organ culture of the mouse mammary gland (MMOC). The mammary glands from balb/c mice are harvested and cultured in presence of appropriate hormones (Mehta et al. 1997b). These glands are subjected to short stimulation with a carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA), which results in formation of precancerous preneoplastic lesions. When implanted in syngeneic hosts, the epithelial cells from these lesions give rise to adenocarcinomas. Effective chemopreventive agents would inhibit the development of these preneoplastic lesions. The chemopreventive activity of a compound in MMOC correlates very well with the activity in in vivo carcinogenesis models (Mehta et al. 1997b). Using a DMBA-induced MMOC model, Mehta et al. (1997a) showed that 1α(OH)D5 possesses chemopreventive activity. Fifteen mammary glands (per group) from balb/c mice were incubated with appropriate hormones and were exposed to the carcinogen DMBA (2 μg/ml of culture media) on day 3 of a 24-day culture. The group of glands incubated with 1α(OH)D5 showed significant reduction of lesion formation compared to the control group (Fig. 3). Percent inhibition of lesion formation in each treatment group was calculated by comparing the incidences of lesions between the control and the treated group. A dose-response curve showed that 100% inhibition was achieved at 10 μM 1α(OH)D5 concentration, but the optimal dose seems to be 1 μM, as it shows significant (75%) inhibition without any signs of cytotoxicity. Vitamin D_3, on the other hand, caused dilation of ducts and disintegration of alveolar structures as signs of toxicity at 1 μM concentration. Based on the MMOC model, 1 μM 1α(OH)D5 seems to be equivalent in potency to 0.1 μM 1α,25(OH)$_2$D$_3$.

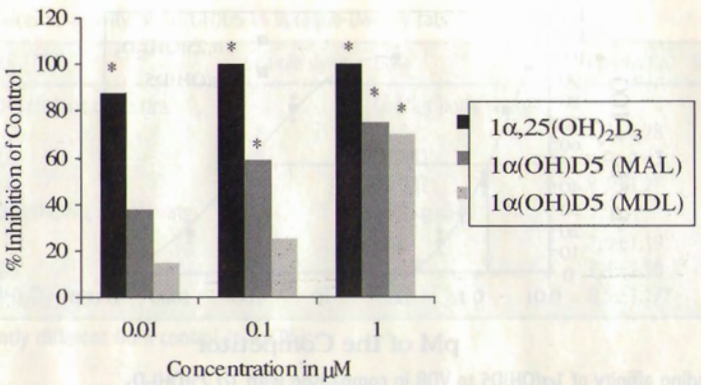

Fig. 3 Chemopreventive efficacy of 1α(OH)D5 in inhibiting mammary alveolar (MAL) and ductal (MDL) lesions in mouse mammary gland organ culture in comparison to 1α,25(OH)$_2$D$_3$

In order to establish the stage specificity for the effectiveness of 1α(OH)D5 in a DMBA-induced MMOC model, 1α(OH)D5 was added either prior to or subsequent to carcinogen treatment. The initiation-only group received 1α(OH)D5 for the first 4 days of culture, whereas the promotion-only group received the treatment after withdrawal of carcinogen (days 4–10). Results indicated that 1α(OH)D5 is more effective when present during the promotional stages of lesion formation (Mehta et al. 2000a). In addition to inhibition of lesion formation, 1α(OH)D5 was effective in inducing VDR and TGFβ1 expression in mammary epithelial cells of MMOC. VDR and TGF-β1 expression was measured using immunohistochemistry. Briefly, paraffin-embedded sections were rehydrated, fixed, permeabilized, and incubated with primary antibody. The primary antibody binding was detected using biotinylated link and peroxidase-conjugated streptavidin, which was then visualized by 3-amino-9-ethylcarbazole as chromogen. The mammary epithelial cells, which stained negative for VDR, failed to show TGF-β1 induction upon 1α(OH)D5 treatment. This implies the involvement of VDR in 1α(OH)D5-mediated effects. The extent of induction of VDR and TGF-β1 upon treatment with 1.0 μM 1α(OH)D5 was similar to that observed with 0.1 μM vitamin D$_3$ (Mehta et al. 1997a). Despite the 1000-fold lower affinity of 1α(OH)D5 for VDR in comparison to 1α,25(OH)$_2$D3, its chemopreventive activity is equivalent to 1α,25(OH)$_2$D$_3$ at only a 100-fold higher concentration. Therefore, it seems likely that the antiproliferative effects of 1α(OH)D5 may not be dependent solely upon its in vitro interactions with VDR.

Since the MMOC experiments involved the whole organ, the actions of 1α(OH)D5 on breast epithelia itself were not clearly established. Hence, we tested the growth effects of 1α(OH)D5 on various breast cancer cell lines of epithelial origin. All the cell lines tested were purchased from ATCC (Manassas, VA, USA), except UISO-BCA-4 cells. This cell line was established in our laboratory from metastatic pleural fluid obtained from a 56-year-old woman with a confirmed diagnosis of breast carcinoma (Mehta et al. 1992). The

Table 2 Growth response of various breast cancer cell lines to 1α(OH)D5 treatment

Cell lines	VDR status	ER status	PR status	Inhibition (%) [a]	Net effect of 1α(OH)D5
BT-474	+	+	+	50	Cell cycle arrest, apoptosis
MCF-7	+	+	+	45	Cell cycle arrest, apoptosis
ZR-75–1	+	+	+	30	Growth inhibition
T-47D	+	+	+	30	Growth inhibition
UISO-BCA-4	+	–	–	40	Growth inhibition, differentiation
MDA-MB-231	–/+ (?)	–	–	None	None
MDA-MB-435	–	–	–	None	None

[a] Percent growth inhibition at 1 μM 1α(OH)D5 for 72 h, adjusted for control

growth effects of 1α(OH)D5 were assessed on BT-474, MCF-7, ZR-75–1, T-47D, UISO-BCA-4, MDA-MB-231 and MDA-MB-435 cell lines using multiple measures: cell counter, MTT absorbance assay (Twentyman and Luscombe 1987), and cell cycle analysis with propidium iodide staining and flow cytometry (Vindelov et al. 1983). The overall effects of 1α(OH)D5 on the growth of different cell lines are summarized in Table 2. All the cell lines that were positive for VDR showed significant growth inhibition ($p<0.05$) after 72 h of incubation with 1α(OH)D5. BT-474, and MCF-7 (VDR+ ER+ PR+) cells showed the greatest growth inhibition and G-1 cell cycle arrest upon 1α(OH)D5 treatment. Similarly, UISO-BCA-4 (VDR+ ER– PR–) cells exhibited growth inhibition in response to 1α(OH)D5 treatment. On the other hand, VDR– MDA-MB-231 and MDA-MB-435 cells did not show any growth inhibition at 1 μM 1α(OH)D5 treatment (Mehta et al. 2002). The dose–response curve for 1α(OH)D5 effect in BT-474 cells was similar to that observed in the MMOC experiments.

Chemopreventive Efficacy of 1α(OH)D5 in In Vivo Carcinogenesis Models

Once we established the in vitro efficacy of 1α(OH)D5, the effects of 1α(OH)D5 were evaluated in experimental mammary carcinogenesis models. We used mammary-specific carcinogen *N*-methyl-*N*-nitrosourea (MNU) in rats to induce tumors and evaluated the efficacy of 1α(OH)D5 to prevent or delay the incidence of mammary cancers in these rats (Mehta et al. 2000a). Fifteen Sprague-Dawley female virgin rats per group (9 weeks old) were fed 1α(OH)D5-supplemented diet (25 or 50 μg/kg) for 2 weeks before the carcinogen treatment. The carcinogen MNU was given as a single intravenous injection of 50 mg acidified MNU/kg body weight at 80 days of age. The rats continued to receive the 1α(OH)D5-supplemented diet until they were killed at 190 days of age. The tumor incidence in control rats was 80%, which, compared to controls, decreased in 25- and 50-μg/kg diet group by 33% and 42%, respectively (Table 3). The tumor incidence in the low-dose group was not sig-

Table 3 Efficacy of 1α(OH)D5 in preventing carcinogenesis in animal models

Tissue	Sample	Dose	Duration[a]	Tumor incidence	Multiplicity
MNU-induced tumors	15	0.0	17	80%	1.6
in rats	15	50 μg/kg diet	17	47%[a]	0.8[a]
DMBA-induced tumors	20	0.0	22	85%	1.9
in rats	20	20 μg/kg diet	22	40%[a]	1.3
UISO-BCA-4 xenograft	5	0.0	6	100%	NA
in athymic mice	5	8 ng (s.c.)[b]	6	0%[c]	NA
UISO-BCA-4 xenograft	5	0.0	6	100%	NA
in athymic mice	5	12.5 μg/kg diet	6	0%[a]	NA
BT-474 xenograft	5	0.0	8.5	0.01 cm^3	NA
in athymic mice	5	12.5 μg/kg diet	8.5	0.125 cm^{3c}	NA

[a] Duration in weeks

[b] 8 ng 1α(OH)D5 subcutaneously injected thrice weekly for 60 days

[c] Significantly different from control ($p<0.05$)

[d] Results are expressed as tumor volume (cm^3)

nificantly reduced from control ($p=0.12$), whereas the high-dose group had a significantly lower tumor incidence ($p=0.03$). However, when the three groups were compared using log-rank analysis, the comparison reached statistical significance ($p=0.0495$). Tumor multiplicity was not significantly different between the control group and the 25-μg/kg diet group, but it was significantly lower in the high-dose group ($p=0.02$).

The encouraging results from MNU-carcinogenesis model prompted us to extend our in vivo experiments. Since MNU is a direct-acting carcinogen, we chose another mammary-specific carcinogen that needs to be metabolized, such as DMBA. For the DMBA carcinogenesis study, 7-week-old rats (20 per group) were given 15 mg DMBA intragastrically. 1α(OH)D5 was supplied in the diet (20–40 μg/kg diet) 2 weeks prior to carcinogen treatment. The control group showed 85% tumor incidence and the high-dose group showed 60% incidence, while the low-dose group showed a significant decrease in incidence (40%). Table 3 summarizes the results from in vivo experiments. Although the high-dose group did not show a significant decrease in tumor incidence, it had significantly lower tumor multiplicity (0.6 compared to 1.9 in the control group). Moreover, the chemopreventive efficacy of 1α(OH)D5 was more pronounced when provided at progressional stages of the disease.

In addition to assessing chemopreventive properties of 1α(OH)D5 in mammary carcinogenesis, we evaluated its efficacy as a possible chemotherapeutic agent. These experiments were carried out in xenograft models, as previously described (Mehta and Mehta 2002). Initial studies were conducted using xenograft of UISO-BCA-4 cells pretreated with 1 μM 1α(OH)D5 for 10 days, which failed to form tumors in athymic (4-week-old) mice. In other studies, UISO-BCA-4 cells were xenografted in athymic mice and either 8 ng 1α(OH)D5 per animal was injected IP thrice a week or 1α(OH)D5 was provided in the diet at

12.5 μg/kg diet for 6 weeks. All the animals in the control group formed tumors whereas only one of the treated animals showed a scab-like structure at injection site in the IP group. Forty percent of controls showed metastasis to lymph nodes but 1α(OH)D5 treatment prevented metastasis of cells transplanted in athymic mice (Mehta and Mehta 2002). In the dietary treatment group, 1α(OH)D5 inhibited growth of UISO-BCA-4 cells and the tumor volume was suppressed to nearly 50% of control. Similar results were obtained with BT-474 xenograft in athymic mice. These results suggest that 1α(OH)D5-induced cell growth inhibition and differentiation is protective against tumor growth in the xenograft model as well.

Growth Response of Normal versus Transformed Cells to 1α(OH)D5

While we established that 1α(OH)D5 has growth inhibitory action on cancer cells, the effects on normal breast epithelial cells were not known. In order to determine that, we cultured mammary glands from mouse with appropriate hormones in the absence of any carcinogens. Ten glands were treated with 1α(OH)D5 and other glands were used as controls. At the end of 6-day culture, the glands were terminated, paraffin embedded, and sectioned for pathological evaluation. Histopathological examination showed no difference in the growth and morphology of glands treated with 1α(OH)D5 from that of control glands. In view this result, we evaluated the effects of 1α(OH)D5 on MCF-12F cells, which are nontumorigenic breast epithelial cells derived from reduction mammoplasty from a 60-year-old Caucasian woman. These cells were spontaneously immortalized by long-term culture in low-Ca^{++} media. To determine their growth response, MCF-12F cells were incubated with 1α(OH)D5 for various intervals, but no growth inhibitory effect was observed at the 1-μM concentration.

To establish selectivity of 1α(OH)D5 effects on transformed or preneoplastic cells, we transformed MCF-12F cells with DMBA and MNU to study if the transformation status could affect the response to 1α(OH)D5. Transformation was performed using the protocol described elsewhere (Lazzaro et al. 1997). Briefly, passage 10 MCF-12F cells were grown to subconfluency in tissue culture dishes and incubated with DMBA (2 μg DMBA/ml culture media) for 24 h. The procedure was repeated the next day. Extensive cell death resulted. The surviving cells were allowed to grow in fresh medium and later selected out with serum starvation. The resulting cell line was designated MCF-$12F_{DMBA}$. Similarly, in another experiment, MNU was dissolved in acidified saline (pH 5.3) and added to subconfluent MCF-12F cells at a concentration of 25 μg/ml twice daily for 2 days. The surviving cells were allowed to grow and the new cell line was established after serum starvation. These cells were called MCF-$12F_{MNU}$. The growth rate and morphological characteristics were compared between these cell lines. The growth rates of transformed cells were three times higher than MCF-12F. By the fifth passage of postcarcinogen treatment, the MCF-$12F_{DMBA}$ doubling time was reduced to one-third of MCF-12F,

Table 4 Growth effects of 1 μM 1α(OH)D5 on normal and transformed MCF-12F breast epithelial cells

Cell line	Treatment	Cell count	Cell cycle (% G-1)	MTT absorbance
MCF-12F	Control	47,250±474	68	0.045±0.06
	1α(OH)D5	45,820±587	71	0.044±0.04
MCF-12F$_{MNU}$	Control	91,800±120	43	0.185±0.06
	1α(OH)D5	73,616±138*	65	0.078±0.01*
MCF-12F$_{DMBA}$	Control	105,470±42.4	49	0.128±0.02
	1α(OH)D5	8,035±91*	67	0.075±0.01*

* Significantly different from control (p<0.05)

while for MCF-12F$_{MNU}$, it was reduced to one-fourth of MCF-12F. Moreover, the transformed cell lines did not exhibit the contact inhibition characteristic of the normal cells.

As mentioned earlier, MCF-12F cells showed no growth inhibitory response with 1α(OH)D5 treatment. The transformed cells, on the other hand, showed significant growth inhibition (60% for MCF-12F$_{MNU}$ and 40% for MCF-12F$_{DMBA}$), as determined by the MTT absorbance assay. Other measures of growth provided similar results (Table 4). These studies indicate that the transformed cells respond differently to 1α(OH)D5 treatment than the parent cell line.

Potential Mechanism of Action of 1α(OH)D5 in Breast Cancer Prevention and Therapy

Previously mentioned studies have implicated the involvement of VDR in 1α(OH)D5-mediated growth effects. VDR− highly metastatic cells such as MDA-MB-231 and MDA-MB-435 do not respond to 1α(OH)D5 treatment. Moreover, mammary epithelial cells which lack VDRs also fail to respond to 1α(OH)D5 and do not show induction of VDR and TGF-β1 (Mehta et al. 1997a). VDR+ breast cancer cells, such as T-47D, had been shown to increase transcription of VDR upon incubation with 1α(OH)D5 as determined by RT-PCR (Lazzaro et al. 2000). This VDR induction was not observed in the cell line BT-474, either at transcription or expression levels, upon treatment with 1α(OH)D5. A possible explanation could be the high constitutive levels of VDR present in this cell line. To ascertain VDR-mediated VDRE transactivation activity of 1α(OH)D5, we used the CAT reporter gene containing VDRE (VDRE-tk-CAT). For this purpose, CV-1 monkey renal cancer cells were used as these lack a functional VDR. After VDRE-tk-CAT transient transfection into CV-1 cells, 1α(OH)D5 could not induce the CAT activity in these cells. But when the cells were cotransfected with VDRE and VDR, there was an enhanced expression of CAT activity, suggesting the capability of 1α(OH)D5 to activate VDR-mediated signaling. The relative CAT activity in CV-1 cells that had been cotransfected with VDRE and VDR was 200,000-fold higher than control when treated with 0.1 μM 1α(OH)D5 (Lazzaro et al. 2000).

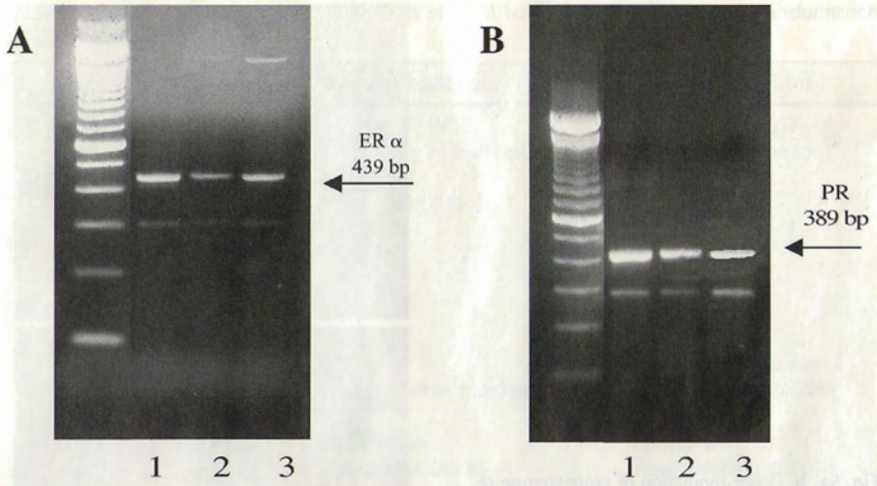

Fig. 4 Down-regulation of estrogen- (**A**) and progesterone- (**B**) receptor transcription with vitamin D_3 and its analog in BT-474 cells as determined by RT-PCR. *Lane 1* control, *lane 2* 1α,25(OH)$_2$D$_3$, *lane 3* 1α(OH)D5

Breast cancer UISO-BCA-4 cells are ER− and PR−, but VDR+. These cells responded differently to 1α(OH)D5 than the ER+ cells (Mehta et al. 2003). UISO-BCA-4 cells were treated with 0.1 μM 1α(OH)D5 for 10 days. The 1α(OH)D5 treatment resulted in induction of intracytoplasmic casein granules, increased lipid droplets, ICAM-1, α2-integrin, nm23, and VDR, manifesting the differentiation markers. Use of this cell line allows us to determine estrogen-independent effects of 1α(OH)D5. While 1α(OH)D5 induced differentiation in ER− cells, it induced apoptosis in ER+ BT-474 and MCF-7 cells, as determined by acridine orange/ethidium bromide staining and TUNEL assay (Mehta et al. 2003). In both these cell lines, there is a G-1 cell cycle arrest followed by apoptosis.

Because the actions of 1α(OH)D5 differ in ER+ breast cancer cells, we examined the effects of 1α(OH)D5 on estrogen-dependent signaling in the ER+ PR+ BT-474 cells. BT-474 cells showed down-regulation of both ER and estrogen-inducible PR transcription upon treatment with 1α(OH)D5, as determined by RT-PCR (Fig. 4). This was in turn followed by down-regulation at the expression level, as estimated by immunocytochemistry (Fig. 5). These results are consistent with reports by other researchers that describe the role of vitamin D_3 in down-regulation of estrogen-inducible genes (Swami et al. 2000; Stoica et al. 1999). The vitamin D_3-VDR pathway may be a negative feedback mechanism to regulate the estrogen-induced proliferation of the mammary tissue. Some researchers have postulated an interaction of VDR-D_3 to ERE to repress the estrogen-mediated gene transcription (Welsh et al. 1998; Demirpence et al. 1994).

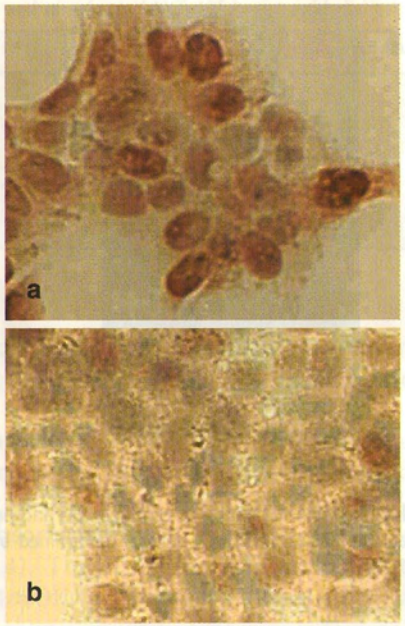

Fig. 5a, b Down-regulation of progesterone receptor (PR) expression with 1α(OH)D5 treatment in BT-474 cells as detected by immunocytochemical analysis. Percentage of cells stained positive for PR were 78% in control (**a**) and 46% in treated cells (**b**)

Since vitamin D_3 is known to regulate a wide variety of genes, we investigated other potential gene targets of 1α(OH)D5 in BT-474 cells. The microarray was performed using Human UniGene 1 by Incyte Genomics, Inc. (Palo Alto, CA, USA), which contained 8,000 genes along with appropriate controls. Among the major targets of 1α(OH)D5 were the estrogen-inducible genes PR, trefoil factor 1 (pS2), and trefoil factor 3 ($p<0.05$). A few selected genes that were statistically significantly altered are presented in Table 5.

As mentioned earlier, the transformed MCF-12F cells showed growth inhibition even though these cells express very low levels of steroid receptors. It is possible that other mechanisms are at work to bring about growth arrest in MCF-12F_{DMBA} and MCF-12F_{MNU} cells. Therefore, we used Clontech Atlas microarrays (Genomics Inc.) with 10,000 genes to identify differentially expressed genes in the transformed MCF-12F_{MNU} cells as compared to the MCF-12F parent cell lines. In a second comparison, we assessed the genes differentially expressed by 1α(OH)D5 treatment in MCF-12F_{MNU} cells. Interestingly, many genes that were differentially expressed in MCF-12F_{MNU} cells compared to the MCF-12F cells were altered inversely in 1α(OH)D5 treated MCF-12F_{MNU} cells (Table 5). Most of the genes that were affected were transcription-related and mitochondrial genes. Of interest are proteins such as vimentin, prohibitin, MAPK-7, and HSP-27, which are usually expressed at higher levels in mammary tumors (Atanaskova et al. 2002; Zajchowski et al. 2001; Storm et al. 1996). These proteins were down-regulated in 1α(OH)D5-treated cells. Differentiation-related proteins such as integrins and cadherins were up-regulated by 1α(OH)D5 in both BT-474 and MCF-12F_{MNU} cell systems.

Table 5 Microarray analysis to determine effects of 1 μM 1α(OH)D5 and MNU-induced transformation on selected genes

Comparison	Genes up-regulated	Genes down-regulated
BT-474 (control)±1α(OH)D5 Incyte Genomics Human UniGene 1 (8 K)	Cytochrome P450 (vitamin D_3 24-hydroxylase) Caspase 3 Cadherin 18 type 2	Trefoil factor 1 (pS2) Progesterone receptor Trefoil factor 3 MMP-9 Thymidine kinase 2 (mitochondrial) Transcobalamine
MCF-12F (control) vs. MCF-12F_{MNU} Clontech Atlas Arrays (10 K)	TGFα Prohibitin Calpain 4 Pituitary tumor transforming 1 HSP-27 Thioredoxin Kearatin 6A and 6B	E2F-4 Integrins Glutathione peroxidase 4 Ornithine decarboxylase antizyme 1 Cystatin B Tissue inhibitor of metalloproteinase 1 TCTP-1
MCF-12F_{MNU} (control)±1α(OH)D5 Clontech Atlas Arrays (10 K)	Glutathione peroxidase 4 ornithine decarboxylase antizyme 1 Cystatin B Tissue inhibitor of metalloproteinase 1 TCTP-1 Integrin β4 Cadherin 3 Cathepsin D	Prohibitin Vimentin MAPK-7 Thioredoxin HSP-27

Prohibitin might be a potentially important vitamin D_3-regulated protein, which was found to be more highly expressed in the transformed MCF-12F cells than the parent cell line (data not shown). Some studies have shown high prohibitin levels in tumor tissue and cancer cell lines (Jupe et al. 1996; Asamoto and Cohen, 1994). However, the role of this mitochondrial protein is controversial. Wang and co-workers (1999) have shown its involvement in regulation of the cell cycle, whereas others have shown that the levels do not represent the cell cycle-related functions but rather are indicative of mitochondrial stress (Coates et al. 2001). It is possible that the mitochondrial stress may be indicative of the higher proliferative rates of the transformed cells. Another protein of interest was thioredoxin, which was up-regulated in MCF-12F_{MNU} cells and down-regulated by 1α(OH)D5 treatment. Thioredoxin is a redox protein with growth factor activity that modulates the activity of several proteins important for cell growth. Some researchers have observed increased thioredoxin transcription and expression in primary human tumors (Matsutani et al. 2001; Berggren et al. 1996). Administration of inhibitors of thioredoxin system has been shown to have antitumor activity in vivo (Kirkpatrick et al. 1999). Moreover, Gallegos and co-workers (1996) reported that transfec-

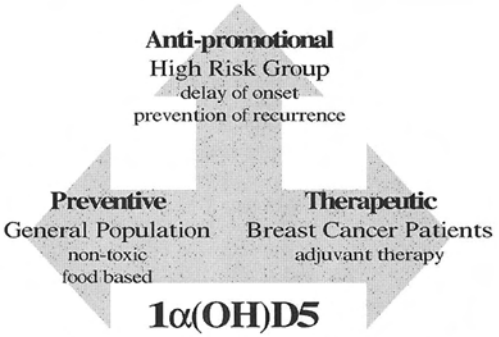

Fig. 6 Potential application of 1α(OH)D5 in breast cancer prevention and therapy

tion of dominant-negative mutant thioredoxin resulted in reversal of transformed phenotype of human breast cancer cells. Therefore, it appears that the mechanism of action of 1α(OH)D5 involves multiple genes and pathways, some of which have not yet been thoroughly investigated. Further studies are needed to elucidate the mechanism of action of 1α(OH)D5 in normal and cancer breast cells.

Conclusions

Results presented in this report on effects of 1α(OH)D5 are suggestive of its promise in chemoprevention. 1α(OH)D5 has consistently been shown to be effective in inhibiting growth of cancer cells as well as preneoplastic lesions in mammary glands in vitro. The in vitro effects are manifested in vivo as well. In the animal carcinogenesis models, 1α(OH)D5 had reduced the incidence of tumors as well as tumor multiplicity, and increased the latency period. Yet there were no changes in total body weight and no apparent signs of toxicity at efficacious doses. More recently, we completed preclinical toxicity studies in rats and dogs under good laboratory practices and regulations, providing an estimation of maximum tolerable dose. The concentration of 1α(OH)D5 required to achieve optimal cell regulatory effects is 100 times higher than the concentration of vitamin D_3. However, there is no hypercalcemia observed at this dose of 1α(OH)D5 to warrant concern. The mechanism of action of 1α(OH)D5 seems to involve VDR as well as cross-talk with the estrogen signaling pathway. It has been shown to inhibit estrogen-induced proliferation. Because of these properties, 1α(OH)D5 might prove suitable in a variety of applications. Furthermore, the differential gene expression profile clearly suggested that the effects of 1α(OH)D5 involve multiple pathways and genes, some of which have not yet been critically studied.

A scheme of possible applications of 1α(OH)D5 is presented in Fig. 6. From a prevention point of view, 1α(OH)D5 might be used in populations that are at high risk or to prevent or delay recurrence of breast tumors in breast cancer

patients. It might also be used in conjunction with other treatments for cancer therapy. Further studies are underway in our laboratory to determine if indeed 1α(OH)D5 would become available for clinical use in the future.

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Chemoprevention of mammary carcinogenesis by 1α-hydroxyvitamin D_5, a synthetic analog of Vitamin D

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Abstract

Numerous analogs of Vitamin D have been synthesized in recent years with the hope of generating a compound that retains the anticarcinogenic activity of Vitamin D without causing any toxicity. We synthesized such an analog, 1α-hydroxy-24-ethylcholecalciferol [1α-hydroxyvitamin D_5 or 1α(OH)D_5], and showed that it was tolerated by rats and mice at a much higher dose than 1α,25 dihydroxy cholecalciferol [1α,25$(OH)_2D_3$]. This property makes it a prime candidate for chemoprevention studies. In the mouse mammary gland organ culture (MMOC), 1α(OH)D_5 inhibited carcinogen-induced development of both mammary alveolar and ductal lesions. In vivo carcinogenesis study showed statistically significant reduction of tumor incidence and multiplicity in *N*-methyl-*N*-nitrosourea (MNU)-treated rats that were fed 25–50 μg 1α(OH)D_5/kg diet. There were no adverse effects on plasma calcium concentrations. In order to determine if the effect of 1α(OH)D_5 would be selective in suppressing proliferation of transformed cells, its effects on cell growth and proliferation were compared between BT474 (cancer) and MCF12F (non-tumorigenic) human breast epithelial cells. Results showed that 1α(OH)D_5 induced apoptosis and cell cycle G1 phase arrest in BT474 breast cancer cells without having any effects on proliferation of the MCF12F cells. In addition, in MMOC it had no growth inhibitory effects on normal epithelial cell proliferation in the absence of carcinogen. Similarly, non-tumorigenic human breast epithelial cells in explant culture did not respond to 1α(OH)D_5, whereas treatment with 1α(OH)D_5 induced cell death in the explants of cancer tissue. These results collectively indicate that 1α(OH)D_5 selectively induced apoptosis only in transformed cells but not in normal breast epithelial cells. Interestingly, the growth inhibitory effects of 1α(OH)D_5 were observed in Vitamin D receptor positive (VDR^+) breast cancer cells, but not in highly metastatic VDR^- breast cancer cells, such as MDA-MB-435 and MDA-MB-231, suggesting that 1α(OH)D_5 action may be mediated, in part, by VDR.

Keywords: Vitamin D; Mammary carcinogenesis; Chemoprevention

1. Introduction

Conceptually, chemoprevention of cancer can be defined as an intervention in the carcinogenic process by either a naturally derived or a synthetic compound. An agent that blocks, arrests, or reverses the progression of cancer can be termed a chemopreventive agent [1,2]. In practice, this can best be achieved by the dietary administration of chemical agents, which can enhance the physiological processes that protect the organism against the development of malignancy. Current understanding of progression of a normal

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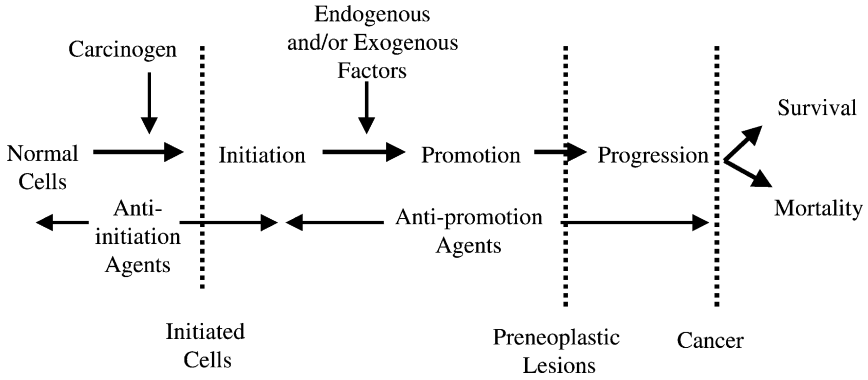

Fig. 1. Schematic diagram to show stages in mammary carcinogenesis and potential points of intervention by chemopreventive agents.

cell to a transformed cancer cell is summarized in Fig. 1. Under experimental conditions, a normal cell could be transformed to an initiated cell in response to carcinogenic or mutagenic stimuli. Although the initiated cells have the potential to develop into malignant cancer, they may or may not form a tumor depending upon exposure to exogenous and/or endogenous factors. In the absence of growth arrest stimuli, the initiated cell can advance to a preneoplastic stage leading progressively to malignancy. The chemopreventive agents that suppress the early events in transformation, such as preventing the mutagenic action of chemicals or other factors, are referred to as anti-initiation agents. On the other hand, chemicals that prevent further progression of initiated cells into transformed ones are termed anti-promotional agents [3,4]. Numerous classes of chemopreventive agents have been reported in the literature, including retinoids, deltanoids, cyclooxygenase inhibitors, inhibitors of polyamine and prostaglandin biosynthesis, lignans, calcium channel blockers, anti oxidants, etc. [5–7]. In this report, we have summarized the chemopreventive properties of a newly evaluated Vitamin D analog, 1-α-hydroxy-24-ethyl-cholecalciferol [1α(OH)D_5].

It has been well established that the active metabolite of Vitamin D, 1α,25-dihydroxyvitamin D_3, [1,25$(OH)_2D_3$] is a steroid hormone and it exhibits potent cell-differentiating properties in leukemia cells as well as other cancer cells of epithelial origin [8,9]. The antiproliferative and differentiation-inducing effects of 1,25$(OH)_2D_3$ could be of clinical significance in prevention or treatment of cancer of several target organs [10]. However, one major limitation in its clinical application is the fact that the efficacious concentrations of 1α,25$(OH)_2D_3$ are cytotoxic [11]. The effective growth inhibitory concentration of 1α,25$(OH)_2D_3$ induces dangerously high levels of serum calcium resulting in loss of body weight and soft tissue calcification, which could be lethal [12]. This has resulted in generation of several non-toxic but antiproliferative synthetic analogs of the Vitamin D molecule for the prevention and treatment of cancer. Some of these analogs have been successfully evaluated for their ability to suppress cancer cell growth in culture as well as in vivo models [13].

Typically, the structure of Vitamin D is divided into four parts (Fig. 2): ring A, open ring B, ring CD, and the side chain. Modifications can be made at all four sites, but the alteration of the ring CD is not common due to its rigid structure. Most alterations have been made at the open side chain. Nearly 800 analogs of Vitamin D have been synthesized so far, and about 300 of them have been evaluated in in vitro and in vivo experimental models [14,15]. Historically, a comparison of the toxicological profile of the Vitamin D series of compounds, including D_2–D_6, had suggested that D_5 was the least toxic of the D series of compounds [16]. In order to generate an effective but non-calcemic and non-toxic Vitamin D analog, we synthesized 1α(OH)D_5 [17]. The structure of 1α(OH)D_5 is shown in Fig. 2.

Vitamin D hormone mediates its action by both genomic and non-genomic pathways. The genomic

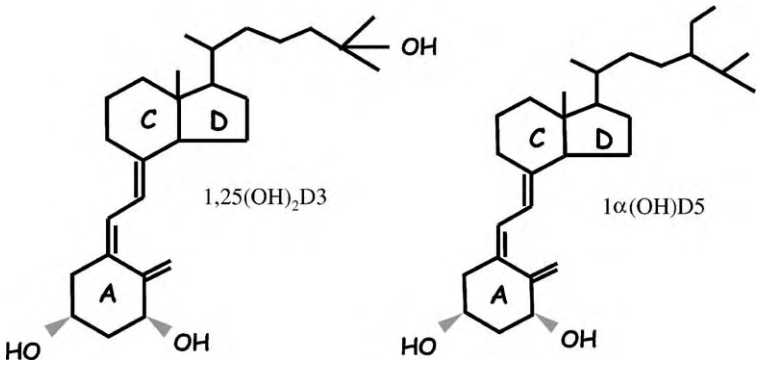

Fig. 2. Structural representation of $1,25(OH)_2D_3$ and its analog $1\alpha(OH)D_5$.

pathway involves its association with high-affinity specific Vitamin D receptor (VDR) that belongs to the steroid receptor superfamily of ligand-activated transcription factors [18–20]. This is consistent with the well-known mode of action of the steroid hormones. The VDR has been identified in a variety of tissues such as breast, prostate, liver, fibroblasts, colon, and lungs [21], in addition to the previously known target organs that included intestine, kidney, and bone.

The VDR mRNA is about 4.6 kb, which translates to a 50-kd protein in humans. The VDR content ranges from 400 to 27,000 copies per cell, yielding 10–100 fmoles/mg of total protein. In order for VDR to function, it needs to bind specific DNA sequences and interact with Vitamin D response elements (VDRE) [22]. The natural metabolite $1\alpha,25(OH)_2D_3$ transactivates VDRE in VDR^+ cells but fails to show interaction in VDR^- cells. Hence, Vitamin D analogs that are able to transactivate VDR–VDRE are mainly mediating their action via genomic pathways. Non-genomic Vitamin D actions have been studied mostly in relation to calcium and phosphorus metabolism, and to a lesser extent with respect to chemoprevention. The rapid responses involve a putative membrane receptor of Vitamin D that signals to modulate calcium channel activity in a cell. This may lead to exocytosis of calcium-bearing vesicles from lysosomes. The non-genomic pathway for Vitamin D action has been extensively reviewed elsewhere [23,24]. For this article, we have listed the chemopreventive properties and possible mode of action of $1\alpha(OH)D_5$.

2. Materials and methods

2.1. Cell lines

We purchased from the American Type Culture Collection (ATCC), Bethesda, MD and maintained in our laboratory according to the ATCC recommendations the following cell lines: (1) the non-tumorigenic, estrogen receptor-negative (ER^-), progesterone receptor-negative (PgR^-), and low VDR breast epithelial cell line MCF12F; (2) ER^+, PgR^+, and VDR^+ breast cancer cell lines BT474 and MCF7; and (3) ER^-, PR^-, and VDR^- breast cancer cell lines MDA-MB-231 and MDA-MB-435.

2.2. Mouse mammary gland organ culture (MMOC)

The detailed procedures for culturing mammary glands from Balb/c mice have been previously reported in the literature [17,25] and outlined in Fig. 3. Briefly, thoracic pairs of mammary glands from Balb/c mice are maintained in serum-free Waymouth's MB752/1 medium under 95% O_2 and 5% CO_2 at 37 °C. The glands respond to growth-promoting hormones insulin, prolactin, aldosterone, and hydrocortisone and differentiate into distinct alveolar structures. Exposure of glands to 7,12-dimethylbenz(a)anthracene (DMBA) for 24 h on day 3 of culture results in the development of precancerous mammary alveolar lesions (MAL). If the growth-promoting medium contains estrogen and progesterone instead of aldosterone and hydrocortisone, the

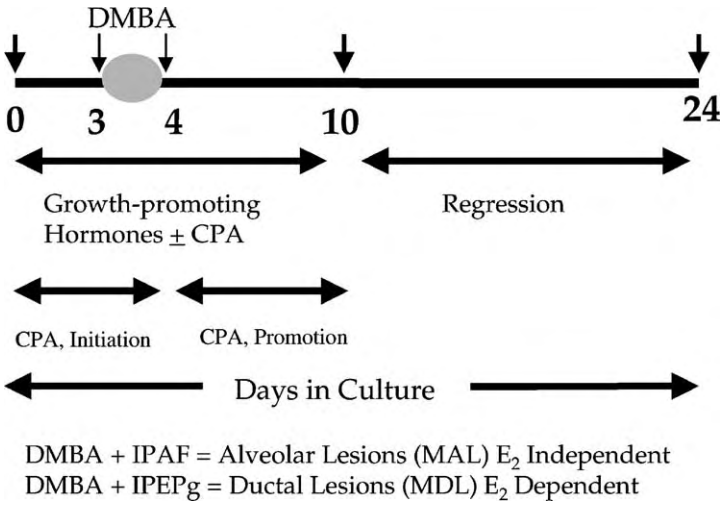

Fig. 3. Experimental design for chemoprevention in mouse mammary gland organ culture (MMOC). DMBA: 7,12-dimethylbenz(a)anthracene, CPA: chemopreventive agent, IPAF: insulin + prolactin + aldosterone + hydrocortisone, IPEPg: insulin + prolactin + estradiol + progesterone, MAL: mammary alveolar lesions, MDL: mammary ductal lesions.

glands develop mammary ductal lesions (MDL) with DMBA treatment [26]. We performed a dose response study to compare the effects of $1\alpha(OH)D_5$ on MAL and MDL. Mammary lesions developed in the absence of $1\alpha(OH)D_5$ served as controls. Additionally, we determined the effects of $1\alpha(OH)D_5$ on normal mammary glands, where the glands were incubated with growth-promoting hormones and 1 μM $1\alpha(OH)D_5$ for 6 days without DMBA treatment. The glands from these MMOC experiments were fixed, stained, and analyzed for morphological characteristics and cell growth and compared with the appropriate controls.

2.3. Cell cycle analysis by flow cytometry

To determine cell cycle, we used flow cytometric analysis as described by Vindeløv et al. [27]. Breast epithelial non-tumorigenic and cancer cells were detached by trypsinization and were harvested. The cells were washed twice with PBS and pelleted. The pellet was resuspended and fixed in 85% ice-cold ethanol. After fixing, the cells were centrifuged and resuspended in citrate buffer and then incubated with NP-40, trypsin, and spermine for 15 min. This was followed by incubation with trypsin inhibitor and RNAase A. The cells were then stained with 0.04% propidium iodide solution. Approximately 10,000 cells were analyzed for DNA content using a Beckman-Coulter EPICS Elite ESP flow cytometer. Multicycle analysis software was used to determine the percentage of cells in various stages of cell cycle. Each experiment was repeated twice and student's *t*-test was used to assess differences.

2.4. Apoptosis

Programmed cell death was evaluated using acridine orange staining. Briefly, a 50 μl suspension of breast epithelial cells was stained with 2 μl of acridine orange/ethidium bromide solution (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS). Cells were layered on a glass slide and examined under a fluorescent microscope with a 40× objective lens using a flourescein filter. Approximately 100 cells were counted on each slide to assess the proportion of cells undergoing apoptosis.

2.5. Mammary carcinogenesis

The procedure for induction of mammary adenocarcinomas by *N*-methyl-*N*-nitrosourea (MNU) in Sprague–Dawley female rats has been described in detail previously [28] and is illustrated in Fig. 4. Briefly, 100-day-old female Sprague–Dawley rats

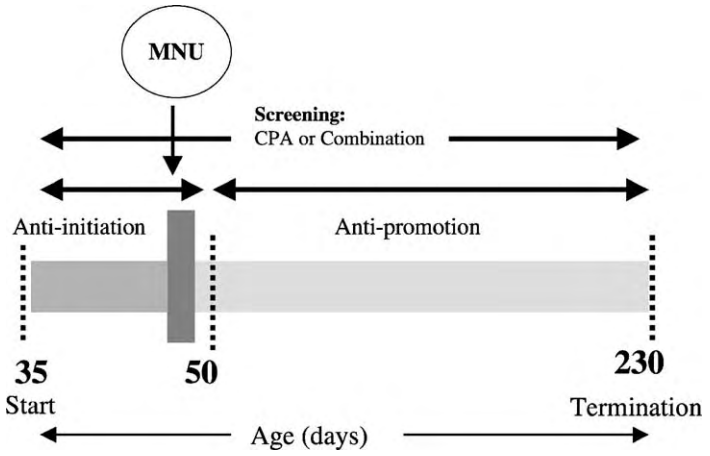

Fig. 4. Schematic diagram to show in vivo model of chemoprevention in *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in Sprague–Dawley rats. CPA: chemopreventive agent.

were injected subcutaneously with 50 mg/kg MNU prepared in acidified saline. Animals received either placebo or 1α(OH)D_5 supplemented as 25 or 50 μg/kg diet. Animals were sacrificed after 230 days of treatment. Mammary tumors were identified by palpation as well as necroscopy. Results were reported as effects of 1α(OH)D_5 on the incidence, multiplicity, and latency of tumor development, and data were subjected to appropriate statistical analyses.

2.6. Effects of 1α(OH)D_5 on normal and malignant breast tissue

Breast tissues were obtained from women undergoing mastectomy or lumpectomy. Explants were maintained in MEME medium, containing 5% stripped fetal bovine serum. The effects of 1 μM 1α(OH)D_5 were determined on these tissues by evaluating cell morphology, apoptosis, and expression of Ki 67. The effects of 1α(OH)D_5 on cell morphology and Ki 67 were compared between the normal and adjacent cancer tissue from the same patient.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Instat® 3.0 software. All MMOC as well as MNU-induced carcinogenesis data were evaluated using χ^2 analysis. Cell viability, apoptosis, and cell cycle results were assessed using two-tailed student's *t*-test with type I error set at 0.05. Serum calcium and phosphorus data were tested with student's *t*-test as well. All in vitro experiments were performed in duplicates and repeated twice.

3. Results and discussion

3.1. Synthesis and toxicity of 1α(OH)D_5

Nearly 300 analogs of 1,25$(OH)_2D_3$ have been evaluated in various experimental systems in the hope of generating analogs that are more efficacious with reduced toxicity. Among the analogs evaluated, only a few have shown potent chemopreventive and therapeutic activity. These analogs include EB1089 [29], KH1060 [30], R024-5531 [31], and 22-Oxacalcitriol [32], which are relatively nontoxic at effective concentrations in experimental models. The hexafluoro analog of 1,25$(OH)_2D_3$, R024-5531, has no calcemic activity, while other analogs do express dose-related calcemia [33,34]. Since it had been reported previously that Vitamin D_5 is the least toxic series of Vitamin D compounds, we synthesized 1α(OH)D_5 with the intention of testing its chemopreventive potential. The chemical synthesis of 1α(OH)D_5 has been previously reported from our laboratory [17].

Since calcemic activity is an obstacle to the development of effective Vitamin D analogs suitable for clinical use, we determined serum calcium and phosphorous concentrations after treating Vitamin D-deficient rats with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$. As reported earlier, male Sprague–Dawley rats (8–10 per group) were fed Vitamin D-deficient diet for 3 weeks, and baseline serum calcium levels were determined. Rats showing <6 mg/dl serum calcium were given $1\alpha(OH)D_5$ for 14 days. Subsequently, serum calcium concentrations were measured. Results showed that $1,25(OH)_2D_3$ significantly ($P < 0.001$) increased serum calcium concentration at a daily dose of 0.042 μg/kg diet, whereas there was no elevation in serum calcium levels among $1\alpha(OH)D_5$-treated animals [17].

A similar experiment was carried out using Vitamin D-sufficient regular diet. Female Sprague–Dawley rats were treated with various concentrations of 1,25 $(OH)_2D_3$ (0.8–12.8 μg/kg diet) and $1\alpha(OH)D_5$ (6.4–50 μg/kg diet) for 2 months. Calcium concentration was increased by 1,25 $(OH)_2D_3$ treatment, while no serum calcium elevation was observed in $1\alpha(OH)D_5$-treated (25 μg/kg diet) animals (Table 1). There was no effect on the final body weight at any dose of $1\alpha(OH)D_5$ used in this study. These results indicate that $1\alpha(OH)D_5$ is considerably less toxic compared to the natural hormone.

More recently, we completed an extensive preclinical toxicity study in both sexes of rats and dogs under good laboratory practice (GLP). Results showed that dogs are relatively more sensitive to the higher dose of $1\alpha(OH)D_5$ than are rats. We concluded from those studies that $1\alpha(OH)D_5$ is calcemic in dogs at concentrations higher than 10 μg/kg diet. The non-calcemic analog R024-5531 shows toxicity in rats without having an effect on serum calcium concentrations. On the other hand, $1\alpha(OH)D_5$ can be tolerated at a higher concentration without other toxicity outcomes.

Table 1
Effects of $1\alpha(OH)D_5$ treatment on serum calcium and phosphorous levels in Sprague–Dawley rats ($n = 10$)

Agent	Dose (μg/kg)	Serum Ca (mg/dl)	Serum P (mg/dl)[a]	BW (% gain)
None		6.3	3.6	100
$1,25(OH)_2D_3$	0.8	7.0	6.4	101
	3.2	7.1	8.0	104
	12.8	7.5*	8.9	70*
$1\alpha(OH)D_5$	6.4	6.3	7.2	99
	12.5	6.2	7.2	97
	25.0	6.5	7.1	98
	50.0	ND	ND	113

* Significantly different from control ($P < 0.05$).
[a] Significance not determined.

Chemoprevention of mammary carcinogenesis by 1α(OH)D5: The chemopreventive properties of $1\alpha(OH)D_5$ have been evaluated in two experimental systems in our laboratory. These include MMOC and MNU-induced mammary carcinogenesis in Sprague–Dawley rats. Mouse mammary glands respond to DMBA and develop preneoplastic mammary alveolar as well as ductal lesions in organ culture. As shown in Fig. 3, the efficacy of a potential chemopreventive agent can be assessed in this assay. If the agent is present and effective prior to carcinogen treatment, its effects are considered as anti-initiation, whereas, if it is effective subsequent to carcinogen, then its effect are anti-promotional. Both types of effects can be determined using the MMOC model.

We showed previously that $1\alpha(OH)D_5$ inhibits the development of mammary lesions in a dose-responsive manner [17]. However, it requires 10-fold higher concentration than the effective concentration of $1,25(OH)_2D_3$. The most effective dose of $1,25(OH)_2D_3$ in suppressing >60% incidence of MAL is 10^{-7} M, while $1\alpha(OH)D_5$ is equally effective at 10^{-6} M without showing cytotoxicity. We also evaluated $1\alpha(OH)D_5$ effects in the MDL model [25]. The results are summarized in Fig. 5. We found $1\alpha(OH)D_5$ to be equally effective against alveolar and ductal lesions.

Since most of the effects of Vitamin D are mediated through VDR, we determined VDR induction by $1\alpha(OH)D_5$ in MMOC as well as in breast cancer cell lines [17]. There was a significant increase in the expression of VDR in the epithelial cells of MMOC as determined by immunocytochemistry. Additionally, $1\alpha(OH)D_5$ also upregulated the expression of TGFβ in the epithelial cells of MMOC [15].

Based on these results, it was reasonable to expect chemopreventive activity of $1\alpha(OH)D_5$ in an in vivo model. Prior to conducting in vivo carcinogenesis studies, a dose tolerance study was conducted in Sprague–Dawley rats. Animals were provided with increasing concentrations of $1\alpha(OH)D_5$, ranging from

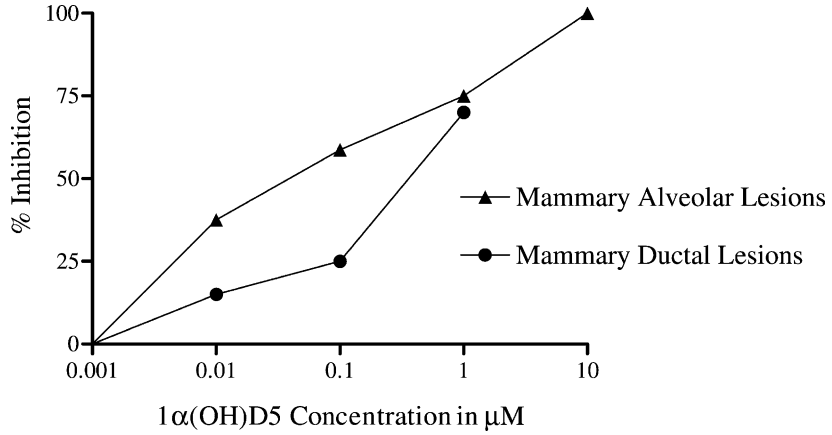

Fig. 5. Effect of $1\alpha(OH)D_5$ on mouse mammary organ culture (MMOC). The glands were incubated with 1 μM $1\alpha(OH)D_5$ for 10 days. The glands were fixed and evaluated for inhibition of preneoplastic lesions in relation to control. Fifteen glands were used per group. A difference in inhibition of greater than 60% was considered significant ($P < 0.05$, χ^2). Data shows significant inhibition of preneoplastic MAL and MDL with $1\alpha(OH)D_5$ treatment.

1 to 100 μg/kg diet for 6 weeks. The animals did not show any adverse effects at any concentration of $1\alpha(OH)D_5$, while the natural hormone was toxic at 3.5 μg/kg diet.

For the MNU-induced mammary carcinogenesis studies, animals were fed $1\alpha(OH)D_5$ at 25 and 50 μg/kg diet for 3 months. The experimental diet was given to the animals 1 week prior to the carcinogen treatment and continued until the end of the study. Results are shown in Table 2. The results indicated a dose-dependent suppression of tumor incidence by $1\alpha(OH)D_5$. This was accompanied by a reduction in tumor multiplicity and an increase in tumor latency [28]. These results are comparable with those of EB1089, R024-5531, and KH1060. The in vivo results as well as the results from MMOC clearly suggest a potential for $1\alpha(OH)D_5$ to be developed as a chemopreventive and therapeutic agent.

Table 2
Chemoprevention of MNU-induced mammary carcinogenesis by $1\alpha(OH)D_5$ in rats

Treatment	Dose (μg/kg)	*n*	Incidence (%)	Multiplicity	Final BW (g)
Control	0	15	80	1.6	228
$1\alpha(OH)D_5$	25	15	53*	1.2	230
$1\alpha(OH)D_5$	50	15	47*	0.8*	226

* Significantly different from control ($P < 0.05$).

3.2. Selectivity of $1\alpha(OH)D_5$ action for transformed cells

We compared the growth effects of $1\alpha(OH)D_5$ in various steroid receptor-positive as well as negative breast epithelial cell lines. These cell lines included (1) non-tumorigenic MCF12F breast epithelial cells, (2) ER^+, PgR^+, VDR^+, BT474, and MCF7 cells, and (3) ER^-, PgR^-, and VDR^- highly metastatic MDA-MB-435 and MDA-MB-231 breast cancer cell lines. The results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ were efficacious in suppressing cell proliferation of ER^+, PR^+, and VDR^+ BT474, T47D, ZR75, and MCF7 breast cancer cells. These compounds induced differentiation of ER^-, PgR^-, VDR^+, and BCA-4 cells [35] but did not show any growth effects in MDA-MB-435 and MDA-MB-231 cells. Other researchers have also reported similar results with other Vitamin D analogs [36]. Although our results indicate that the presence of VDR is necessary to potentiate Vitamin D's effect, it does not explain the lack of Vitamin D's effect on MCF12F cells that express low levels of VDR.

In order to examine whether $1\alpha(OH)D_5$ selectively inhibits cell proliferation in transformed cells only, we evaluated the effects of $1\alpha(OH)D_5$ on non-tumorigenic breast epithelial cells and compared them to the effects on BT474 breast cancer cells.

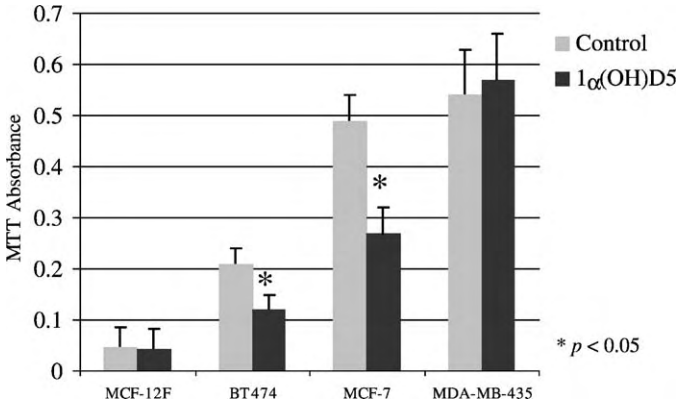

Fig. 6. Effects of $1\alpha(OH)D_5$ on viability of non-tumorigenic and cancer breast epithelial cells. Different cell lines were treated with 1 μM $1\alpha(OH)D_5$ for 2 days and incubated with MTT for 2 h. The cells were lysed and washed prior to reading absorbance at 550 nm. MTT absorbance is proportional to the number of live cells. Each experiment was repeated twice and differences between the mean were assessed using student's *t*-test.

As shown in Fig. 6, incubation of MCF12F breast epithelial cells for 6 days with $1\alpha(OH)D_5$ at 1 μM concentration did not result in suppression of cell proliferation as determined by the MTT absorbance assay. On the other hand, there was a significant inhibition of proliferation in both MCF7 and BT474 cells with $1\alpha(OH)D_5$ treatment. These results suggested that the effect of Vitamin D analog might be selective for transformed cells. The antiproliferative effects of $1\alpha(OH)D_5$ were also evident in in vivo experiments. Xenograft of ER^+, PgR^+, VDR^+, MCF7, ZR75/1, and BT474 cells or ER^-, PgR^-, VDR^+, and BCA-4 cells responded to 12.5 μg $1\alpha(OH)D_5$/kg diet and showed suppressed growth of these cells in athymic mice [35].

To confirm the selectivity of $1\alpha(OH)D_5$ for transformed breast cancer cells, we conducted three separate experiments. In the first experiment, we compared the efficacy of $1\alpha(OH)D_5$ between MCF12F cells with that of MNU-transformed MCF12F ($MCF12F_{MNU}$) cells. The $MCF12F_{MNU}$ cells have recently been established in our laboratory (unpublished data). The $MCF12F_{MNU}$ cells have altered morphology and growth properties as well as different growth factor requirements (Hussain and Mehta, unpublished data). Incubation of MCF12F and $MCF12F_{MNU}$ with 1 μM $1\alpha(OH)D_5$ for 6 days resulted in 50% growth inhibition in $MCF12F_{MNU}$ cells without having any significant effects on MCF12F growth.

In a second study using the MMOC model, the effects of $1\alpha(OH)D_5$ were determined in mammary glands. Mammary glands respond to growth-promoting hormones and develop structurally differentiated alveoli within 6 days in culture. Incubation of glands with 1 μM $1\alpha(OH)D_5$ for 6 days did not affect the growth-promoting effects of insulin, prolactin, aldosterone, hydrocortisone, estrogen, and progesterone (Fig. 7). Contrarily, $1\alpha(OH)D_5$ showed excellent anti-proliferative effects against DMBA-induced MAL and MDL (Fig. 5).

Experiments to determine the selectivity of $1\alpha(OH)D_5$ action against transformed cells were further extended to human tissues. The effects of $1\alpha(OH)D_5$ on the explants derived from normal breast tissues were compared with those of cancer tissue. Breast tissue samples were obtained from women undergoing mastectomy or lumpectomy at the University of Illinois at Chicago Hospital. Tissue explants of tumors and normal adjacent cells were incubated for 72 h in the MEME containing 5% fetal calf serum with or without $1\alpha(OH)D_5$ at 1 μM concentration. Tissue sections were histopathologically evaluated, and Ki 67 expression was determined. Results showed that the histopathology of control and $1\alpha(OH)D_5$-treated normal breast tissue was identical with no difference in apoptosis or Ki 67 expression. On the other hand, the histological sections of the cancer tissue explants showed extensive apoptosis within the tissue with

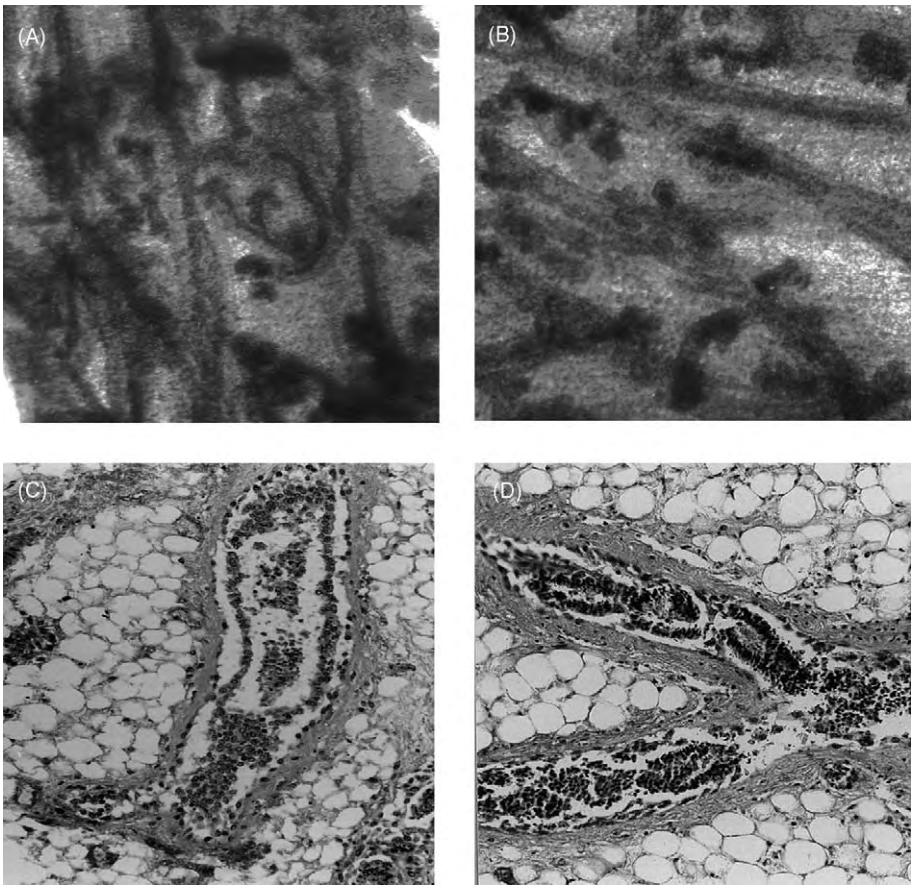

Fig. 7. The 6-day mouse mammary organ culture (MMOC) was performed without the carcinogen treatment. The data shows similar growth in both the control and 1α(OH)D_5 treated glands. (A) control; (B) 1α(OH)D_5; fixed and stained with carmine; (C) control and (D) 1α(OH)D_5, fixed, sectioned, and stained with H and E.

condensed chromatin and reduced Ki 67 expression after 72-h incubation with 1α(OH)D_5 (Mehta, unpublished data). Taken together, these results indicate that, in human breast epithelial tissues, 1α(OH)D_5 is selective for its effects on pre-cancerous or cancer cells but shows no effect on normal breast epithelial cell growth.

3.3. Mechanism of 1α(OH)D_5 action

The effects of 1α(OH)D_5 have also been evaluated in several breast cancer cell lines [37]. Although these studies do not focus directly on chemoprevention, they do provide excellent insight into the mechanism of action of 1α(OH)D_5 and its efficacy as an anti-proliferative agent. We had reported that, in ER^+, PgR^+, breast cancer cells, 1α(OH)D_5 inhibited cell growth by inducing apoptosis as well as differentiation, whereas in ER^- but VDR^+ cells, it induced cell differentiation without the induction of apoptosis [35]. Similar results have also been reported by numerous investigators using other analogs of Vitamin D [38]. The data from these studies consistently reported that breast cancer cells expressing VDR respond to Vitamin D analogs. These results suggested that the mode of action of 1α(OH)D_5 depended not only on expression of VDR but also on the expression of ER and ER-inducible genes such as PgR.

The effects of 1α(OH)D_5 on cell cycle were determined using breast cancer cells. The BT474 cells

Table 3
Effects of $1\alpha(OH)D_5$ on cell cycle phases in breast epithelial cell lines

Types	G1 (%)	S (%)	G2 (%)	G1/G2 (%)
BT474				
Control	60.7	30.5	8.8	6.9
$1,25(OH)_2D_3$	71.6*	22.1	6.3	11.4
$1\alpha(OH)D_5$	85.7*	10.3	4.0	21.4
MCF7				
Control	61.2	28.6	10.1	6.1
$1,25(OH)_2D_3$	71.9*	19.3	8.8	8.2
$1\alpha(OH)D_5$	70.0*	20.4	9.6	7.3
MDAMB435				
Control	22.8	31.3	45.9	0.5
$1,25(OH)_2D_3$	21.1	33.0	45.3	0.5
$1\alpha(OH)D_5$	21.1	23.6	55.3	0.4
MCF12F				
Control	72.4	16.2	11.4	6.4
$1,25(OH)_2D_3$	61.1*	20.2	19.0	3.2
$1\alpha(OH)D_5$	67.3*	16.2	16.5	4.1

* Significantly different from control ($P < 0.05$).

were treated with 1 μM $1\alpha(OH)D_5$ for various time points and processed for FACS analysis. Results showed that 70% of the control cells were distributed in the G1 phase, whereas treatment with $1\alpha(OH)D_5$ induced growth arrest with 84% cells in the G1 phase of the cycle. The results are summarized in Table 3. In agreement with our cell proliferation data, there was no difference between the distribution of cells in various cell cycle stages for MCF12F and MBA-MD-231 cells with $1\alpha(OH)D_5$ treatment. Both MDA-MB-231 and MDA-MB-435 cells are devoid of steroid receptors; therefore, these cells were not expected to respond to $1\alpha(OH)D_5$ treatment. These results further confirm that the action of $1\alpha(OH)D_5$ may be mediated, in part, by VDR.

The mechanism of action of $1\alpha(OH)D_5$ was further evaluated by determining the ability of the cells to undergo apoptosis. The BT474 cells were treated with $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 72 h and then stained with acridine orange and observed under fluorescent microscope for detection of chromatin condensation. Fig. 8 shows that BT474 cells underwent apoptosis with $1\alpha(OH)D_5$ treatment as determined by acridine orange and ethidium bromide staining. The stain distinguishes live cells from those that are undergoing apoptosis. On the other hand, no apoptosis was observed in ER^-, PgR^-, VDR^+, BCA-4 cells, though there was an induction of differentiation as shown by casein, lipids, and α2 integrin expression [35].

Chemopreventive agents are being developed mostly for people who do not yet have disease but are at high risk of developing cancer. Here, we show that the Vitamin D analog might be selective for transformed cells. The population at high risk of developing cancer is assumed to be initiated for carcinogenesis and, as we have shown, initiated cells respond well to $1\alpha(OH)D_5$. In addition, we also showed here that $1\alpha(OH)D_5$ is effective against steroid-responsive cancer cells. These results suggest that $1\alpha(OH)D_5$ can be considered as a possible chemopreventive and therapeutic agent. Moreover, if given in combination with other agents, it may provide synergistic protection.

It is unclear as to where chemoprevention ends and chemotherapy begins. However, the clear principle

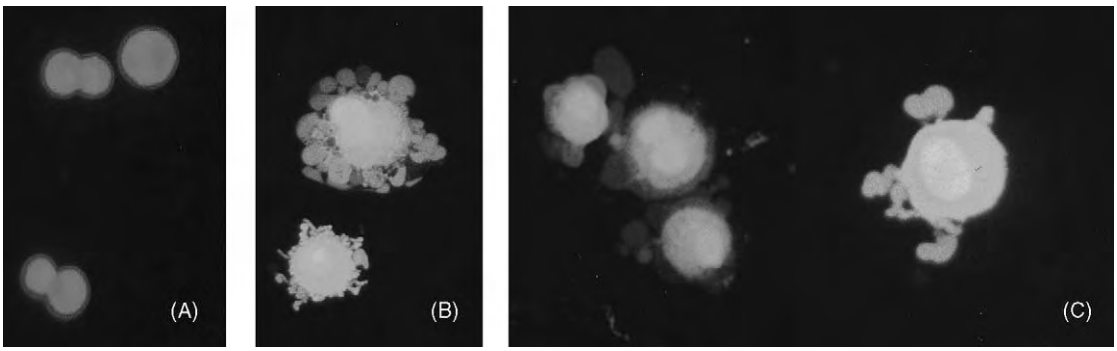

Fig. 8. Induction of apoptosis in BT474 cells by 1 μM $1\alpha(OH)D_5$, as determined by acridine orange and ethidium bromide staining. (A) control; (B) $1,25(OH)_2D_3$ (0.1 μM); (C) $1\alpha(OH)D_5$ (1 μM).

and prerequisite of chemoprevention is that the agent should not have any adverse effects. The lack of toxicity of $1\alpha(OH)D_5$ at an effective concentration may provide a rationale for its role in chemoprevention and therapy.

In summary, we have described here the chemopreventive properties of a relatively new non-toxic analog of Vitamin D, $1\alpha(OH)D_5$, against mammary carcinogenesis models. In addition, our results suggest that $1\alpha(OH)D_5$ may be active selectively against transformed cells without showing adverse effects on normal breast epithelial cells.

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EFFECT OF VITAMIN D ANALOG (1α HYDROXY D5) IMMUNOCONJUGATED TO Her-2 ANTIBODY ON BREAST CANCER

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We previously showed that a new vitamin D analog, 1α(OH)D5 (D5), induced differentiation and inhibited the growth of breast cancer cells. In this report, we examined whether D5 specifically delivered to breast cancer cells could have any therapeutic effect. D5 was linked to Her-2 antibody using sulfosuccinimidyl 6-4 azido nitrophenylamido hexanode (SANPAH) as a linker. The Her-2 antibody selected in our study had no significant effect on the *in vitro* or *in vivo* growth of breast cancer cells; however, it had cell-differentiating action. *In vitro*, D5-Her-2 antibody conjugate (IMC) showed the ability to specifically bind to Her-2-expressing cells, to compete with Her-2 antibody for surface receptor and to cause internalization. IMC (equivalent to 5 μg Her-2 antibody given intraperitoneally once weekly for 6 weeks) significantly inhibited the growth of BT-474 cells transplanted into athymic mice. The *in vivo* growth-inhibitory effect of IMC treatment was similar to that observed in animals receiving D5 continuously as a dietary supplement. These results show that the targeted delivery of D5 by immunoconjugation to cell surface receptor antibodies may be of potential therapeutic value for the treatment of Her-2 positive breast cancer.

Key words: *vitamin D; analog; immunoconjugate; Her-2*

Even though recent introduction of breast screening mammography and highly sensitive diagnostic procedures has helped in the early diagnosis of breast cancer, control of metastatic disease remains a major concern of oncologists.[1] Many breast cancers, especially those showing overexpression of Her-2 protooncogene, are more aggressive and are characterized by early metastatic spread and reduced patient survival.[2–6] Importantly, overexpression of Her-2 protein is associated with altered clinical responsiveness to some standard chemotherapy regimens.[2,7,8] Thus, new therapeutic approaches are urgently needed for treating highly aggressive Her-2-overexpressing breast cancers. Special emphasis has been given to the search for the agents that have both antiproliferative and cell-differentiating actions on breast cancer cells. Vitamins, especially the vitamin D metabolite 1,25(OH)D3, have been shown to *(i)* suppress the development and progression of breast cancer and other carcinomas *in vivo* and *in vitro*; *(ii)* inhibit the metastatic spread of tumor cells and *(iii)* promote differentiation of breast cancer cells.[9–16] However, the concentration necessary for the growth inhibitory effect on cancer cells *in vivo* causes hypercalcemia.[17,18] Thus, the recent focus is to identify highly effective but nontoxic vitamin D derivatives.

We showed earlier that a novel vitamin D analog (D5) has potent cell-differentiating and antiproliferative actions in breast cancer cells.[15,16] *In vitro*, after 7 days exposure of breast cancer cells to 0.1–1 μM D5, we observed the induction of various biomarkers associated with cell differentiation, such as an increase in intracellular accumulation of neutral lipid, an increase in intracellular casein and the enhanced expression of α2 integrin.[15,16] Human breast cancer cells exposed *in vitro* to D5 also lost their tumorigenic potential when transplanted into athymic mice.[15]

Interestingly, D5 supplemented in the diet inhibited *in vivo* growth of breast cancer cells in athymic mice.[15] D5 also reduced the incidence of carcinogen-induced mammary tumors in the experimental rat model.[19] In our study, we examined the possible therapeutic potential of targeted delivery of D5 to Her-2-overexpressing breast cancer cells by using an immunoconjugate linked to the Her-2 antibody in an experimental model.

MATERIAL AND METHODS

Human breast carcinoma cell lines

The breast carcinoma cell line BT-474 was obtained from the American Type Culture Association (ATCC; Rockville, MD). The cells were maintained in MEM-E (minimum essential medium containing Earl's salt) supplemented with 10% FBS, essential amino acids, glutamine and streptavidin fungizone.

Vitamin D analog and the Her-2 antibody

D5 was purchased from Onquest, Inc. (Chicago, IL). For both *in vitro* and *in vivo* studies, D5 was originally dissolved in 100% ethanol. For *in vitro* studies, stock solution was diluted and then added to the culture medium so that the final concentration of ethanol in the medium was less than 0.01%. Antibody (BSA-free, azide-free) against the extracellular domain of Her-2 receptor used for both *in vivo* and *in vitro* studies was obtained from Neomarkers (Fremont, CA). The Her-2 antibody (clone 9G6.10, ab-2) is reported to immunoprecipitate 160 KDa protein from extracts of Her-2-positive cells.[20] The antibody has no growth-inhibitory action *in vivo* in the breast cancer xenograft model. Control immunoglobulin (Ig)G isotype matched antibody (mouse IgG, clone NC G01, BSA-free, azide-free) was obtained from Neomarkers (Fremont, CA).

Preparation of the D5-Her-2 antibody immunoconjugate

D5 was covalently linked to antibodies (Her-2 antibody or control antibody) using sulfosuccinimidyl 6-4 azido nitrophenylamido hexanode (SANPAH; Pierce, Rockford, IL) as a linker. Immunolinking of D5 was performed by the 2-stage method based on photoaffinity crosslinking. In brief, the crosslinking agent SANPAH was first coupled to the amino group of the antibody. The antibody (1 mg/ml = 6.67 μM final concentration) was incubated in the dark with different molar concentrations of SANPAH as a linker (133.4–533.6 μM final concentration). After incubation, unreacted SANPAH and hydrolyzed linker by-product were removed by G-25 sephadex gel filtration. Different molar concentration of D5 (133.4–533.6 μM final concentration) was added to the reaction mixture and subjected to photo activation by 3–4 camera flash light exposures. The nitro-substituted aryl-azide,

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when photolysed, forms an aryl nitrene that reacts nonselectively with D5 to form a covalent bond. Excess vitamin D analog was removed by dialysis. Unconjugated antibody was not removed from the reaction mixture. Each preparation was tested for the specific binding ability to Her-2 receptor on BT-474 cells. The immunoconjugate (IMC) preparation was loaded on a 4–20% SDS polyacrylamide gel. After being electrophoresed, the gel was exposed overnight to Kodak radio autography film in the dark at 4°C. If nonradioactive antibody was used in the IMC preparation, the SDS-PAGE gel was immunoblotted on immunobiolon paper, subjected to secondary anti-mouse IgG conjugated to horseradish peroxidase (HRP) and then subjected to chemiluminescence analysis using a kit from Amersham Pharmacia Biotech (Piscataway, NJ). All batch preparations were tested for electrophoretic mobility before use in the experiments.

Competitive binding of IMC with the Her-2 antibody

BT-474 cells were seeded at 20,000 cells/well density in 96-well plates. Cells were allowed to attach to the culture wells overnight. Cells were washed twice with PBS and were fixed in 0.06% gluteraldehyde. After washing with PBS, cells were incubated for 10 min in PBS containing 10% FBS to reduce the nonspecific binding. Cells were then incubated with ^{125}I-labeled Her-2 antibody (S.A. = 1.8 μCi/μg, 10 ng) or ^{125}I IMC (S.A. = 1.2. μCi/μg, 10 ng) alone or in the presence of increasing concentrations (0–750 ng) of unlabeled Her-2 antibody. ^{125}I antibody was aliquoted in the wells without cells and was processed along with samples as a blank. We also incubated the ^{125}I-labeled Her-2 antibody with increasing concentrations of the control antibody or with its IMC with D5. The cells were incubated at RT for 2 h and then washed with PBS twice. Radioactivity was extracted by incubating the cells in PBS containing 1% SDS at room temperature for 15 min. Liquid was absorbed from the wells using cotton swabs, and the radioactivity in the cotton swabs was counted using a gamma counter. Radioactivity detected in the blank wells was considered as nonspecific counts and was subtracted from binding detected to the cells in the presence/absence of unlabeled antibody. Data represent percent binding in the absence of unlabeled antibody. Each data point is a mean value ± standard error (SE) of 3 independent observations.

Internalization of IMC

For the internalization assay, BT-474 cells growing on sterile glass cover slips were incubated with 1.5 μg/ml Her-2 antibody or control antibody alone (in medium containing 5% charcoal-stripped FBS) or IMC (D5 linked to control or Her-2 antibody, equivalent to 1.5 μg/ml antibody) at 37°C for 0–24 h. At the end of incubation, cells were washed extensively with PBS, fixed in 10% buffered formalin and then permealised in cold methanol, washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Dako Corp., Carpinteria, CA) for 30 min. After washing, cells were coverslipped using vectashield aqueous mounting media containing DAPI (4, 6-diamidino-2-phenylindol, 1.5 μg/ml; Vector Laboratories, Inc., Burlingame, CA). Fluorescence images were obtained using a Carl Zeiss LSM 510 laser scanning confocal microscope (BioRad, Richmond, CA) equipped with a X63 water emulsion objective. Beams of 488 nm and 364 laser were used for excitation and blue (DAPI) and green (FITC) fluorescence were recorded through LP505 and LP470 filters, respectively. For quantitative analysis, cells growing in a monolayer were incubated for 0–24 hr in the presence of the Her-2 antibody and IMC, washed with PBS, fixed in 0.5% buffered formalin and then treated with ice cold methanol for 5 min. After PBS wash, the cells were incubated with FITC-labeled secondary antibody for 30 min and then washed with PBS. Relative fluorescence intensity was determined by fluorescence activated cell sorting (FACS) analysis (Epic Elite Flow Cytometer, Coulter Corporation, Miami, FL). Data are presented as fluorescence intensity recorded in arbitrary units.

Immunostaining for Ki-67 and intracytoplasmic casein

To determine the *in vitro* effect on cell proliferation, Ki-67 immunostaining was used as a biomarker. For differentiation, accumulation of casein granules was determined immunohistochemically in the cells. BT-474 cells were plated on Nunc culture cover slips and allowed to attach to the culture wells overnight in MEM-E containing 10% FBS. The next day, the culture medium was replaced with SS medium (MEM-E containing 5% charcoal-stripped FBS) containing ethanol as a vehicle, 1 μM D5 (428 ng/ml), Her-2 antibody (1.5 μg/ml), control antibody (1.5 μg/ml), or IMC of respective antibodies (1.5 μg Her-2 antibody/control antibody containing 171.2 ng of D5 assuming that all D5 used in IMC preparation gets immunoconjugated). The cells were incubated at 37°C in the atmosphere of 95% air and 5% CO_2. Medium was changed with respective ingredients on day 4 after initiation of the treatment. The cells were thoroughly rinsed in PBS and fixed in 10% buffered formalin followed by ice-cold methanol. The cells were first incubated with anti-mouse FITC-labeled antibody to avoid interference of Her-2 antibody/immunoconjugate preincubation in Ki-67 staining. Ki-67 staining was performed in fixed cells by the method described previously.[15,16] Cells were counterstained using hematoxylin. Only AEC (3-amino-9-ethylcarbazole; Biogenex, San Ramon, CA) staining in the nucleus was considered positive; the number of positive cells/total number of cells was counted in 10 different high-power fields, and the percentage of cells positive for Ki-67 were calculated. The experiments were repeated using 10 different cover slips in each treatment group, and the data represent percent mean ± SE in a minimum of 10 different cover slips.

For casein staining, the cells were incubated with rhodamine-labeled anti-mouse secondary antibody to prevent the interference from the Her-2 antibody preincubation in casein immunostaining. The cells were washed thoroughly with PBS, incubated with anti-casein antibody (Neomarker, Freemont, CA; 1:100 dilution), incubated with FITC-labeled secondary antibody (Dako, Carpinteria, CA) and visualized under a microscope.

Lipid staining in BT-474 cells

The cells cultured on the cover slip were treated as described above. After incubation, the cells were immediately washed with Hepes (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, 1M)

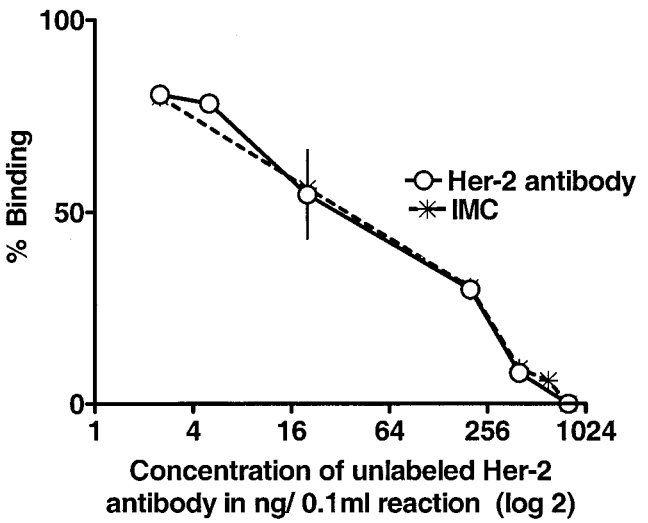

FIGURE 1 – BT-474 cells (20,000 cells/well) were incubated for 2 h at room temperature with ^{125}I-labeled Her-2 antibody or ^{125}I Her-2 1α(OH)D5 IMC alone or in the presence of increasing concentrations of unlabeled Her-2 antibody. After PBS wash, radioactivity was extracted and counted using a gamma counter. Each data point represents the mean ± SE of 3 independent observations.

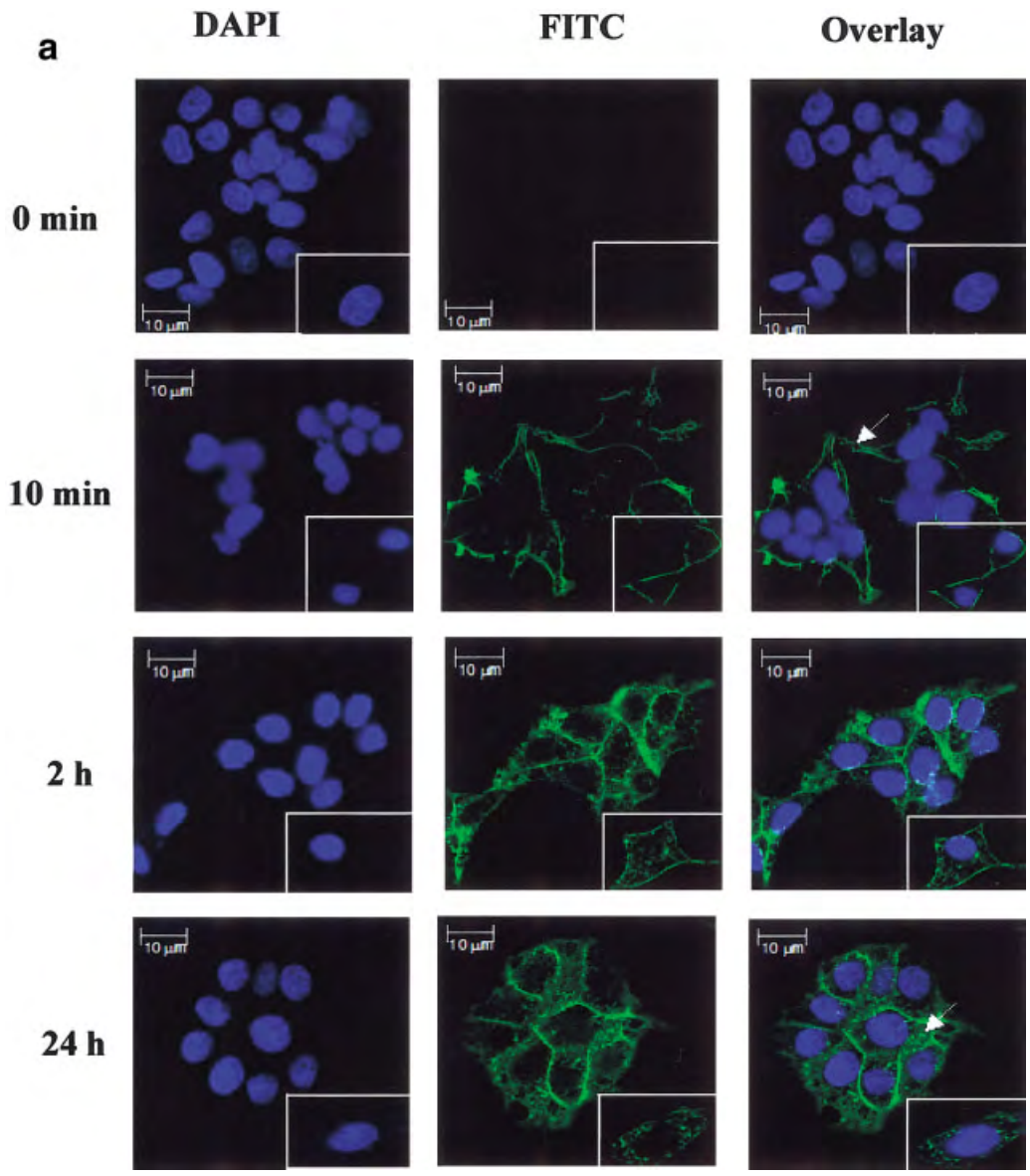

FIGURE 2 – BT-474 cells were incubated with (1.5 μg/ml) Her-2 antibody *(a)* or IMC *(b)* at 37°C for 0–24 h. At the end of incubation, cells were washed, fixed in 10% buffered formalin and permealised in cold methanol, then incubated with FITC-conjugated anti-mouse IgG. The cells were counterstained with DAPI and the images were taken using a confocal laser scanning microscope. Insets represent cropped images of the isolated cells. Arrow shows cell surface (10 min) or intracellular vesicular (24 h) immunostaining. Quantitative analysis of Her-2 antibody and IMC in BT-474 cells *(c)*. Cells were incubated at 37°C with 1.5 μg/ml Her-2 antibody or IMC for 0–24 h. Accumulation of Her-2 antibody or IMC in the cells was determined by incubating cells with FITC-labeled anti-mouse IgG. Relative fluorescence intensity was determined using a flow cytometer. The bar graph shows representative data of 3 independent experiments. Data represent mean ± SE.

buffer and then stained with Nile Red (Sigma Chemical Co., St. Louis, MO; 1 mg/ml in acetone, diluted to 1:200 in Hepes buffer) stain for 5 min. The cells were immediately visualized under fluorescence microscope (using a blue filter) and photographed. To further confirm that the staining observed was due to neutral lipids, a set of coverslips was rinsed in isopropyl alcohol before staining with Nile Red stain.

The effect of Her-2 antibody, D5 and IMC on in vivo *growth in breast cancer cells*

Animals were obtained from Frederick Cancer Research Facility (Bethesda, MD). BT-474 cells (1 million cells/animal) were suspended in a mixture (1:1 vol./vol.) of HBSS (Hank's balanced salt solution; Invitrogen Corporation, Carlsbad, CA) and Matrigel (BD Sciences, Bedford, MA), then injected subcutaneously into the dorsal flank region of 3- to 4-week-old female Balb/c athymic mice. All animals received a subcutaneous estrogen pellet (0.72 mg/animal, 60 days release; Innovative Research, Saratoga, FL). Once the palpable tumor developed (0.03 cm^3), animals were divided into various groups: *(i)* receiving regular powdered diet mixed with ethanol as vehicle (control diet); *(ii)* receiving D5 (12.5 μg/kg diet)-supplemented powdered diet; *(iii)* receiving intraperitoneal injection of Her-2 antibody (5 μg/animal, once weekly) and regular powdered mouse diet as in the first group; *(iv)* receiving IMC (equivalent to 5 μg Her-2 antibody) once weekly intraperitoneally and receiving the control diet. Because we did not observe any effect of control antibody and control antibody IMC on *in vitro* growth of BT-474 cells, these treatments were not further tested in *in vivo* study. D5 contents in various *in vivo* administrations are

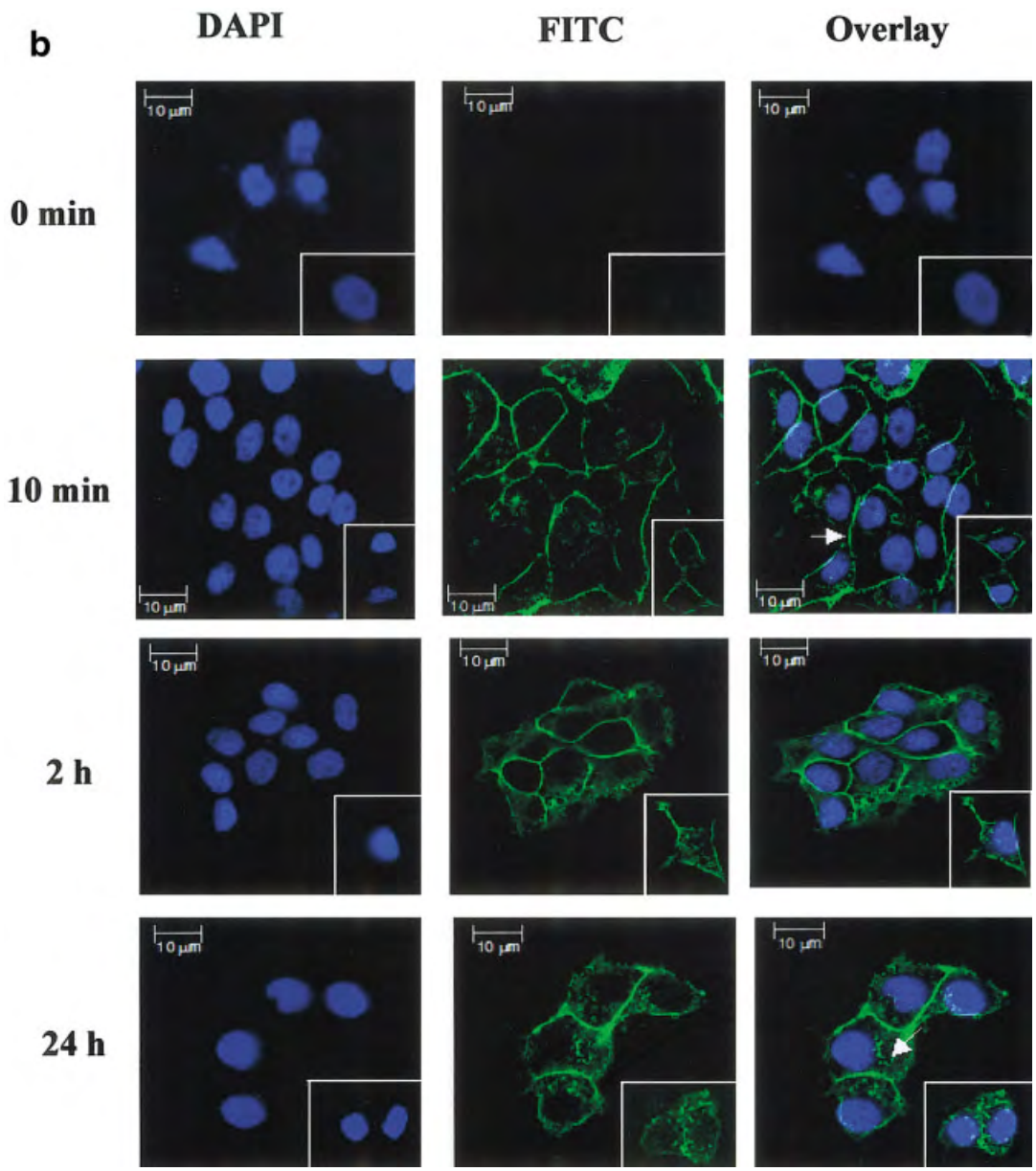

FIGURE 2 – CONTINUED.

estimated as follows. Dietary D5 was given at 12.5 μg/kg dose. On average, diet consumption by an athymic mouse is between 6–7 g/day. Thus, the amount of D5 consumed by an animal each day ranges between 75–87 ng/day or 525–621 ng/week. For IMC treatment, 5 μg IMC contains (assuming 100% of D5 added to 1 ml of antibody gets immunolinked) 570.9 ng D5. IMC was further diluted with HBSS to get a final injection dose of 5 μg IMC/100 μl HBSS). In a separate experiment, we also tested whether intraperitoneal administration of D5 (D5 stock solution was made to obtain 266.8 μM final concentration and then further diluted to give 570.9 ng/ml D5 for injection into animals) given once weekly for 4 weeks has any effect on the growth of BT-474 xenograft.

Both the control and D5-supplemented diets were given to the animals in sterile food cups; an equal amount of food was placed in each cup. Food cups were protected from direct light exposure. Food cups were changed twice weekly. For preparation of D5-supplemented diet, a known amount of D5 was dissolved in absolute ethanol and then mixed with powdered mouse chow (Teklad, Madison, WI) using a diet mixer. Diet was stored in foiled containers to protect from light, and stored at 4°C. The stability of D5 was determined periodically.

An aliquot of the diet was extracted with methanol, and the extract was subjected to high performance liquid chromatography (HPLC) analysis. The control diet was mixed with ethanol (equal to that used for D5 diet) only. Ethanol from the diet mixtures was evaporated by placing it at room temperature for 20 min in sterile culture hoods. All animals received water *ad libitum*. Each group consisted of a minimum of 5 animals. The experiment was repeated twice. The animals were examined once weekly for growth of tumor at the site of injection, and tumor size was monitored using calipers. Tumor volume was calculated as cm^3 using the following formula: tumor volume (cm^3) = 3.14/6 × length × width × depth. Data represent the mean tumor volume ± SE (cm^3) in each group. The animals were sacrificed at the indicated time unless they appeared to be moribund or tumors showed sign of necrosis. At termination, blood was collected for calcium determination.

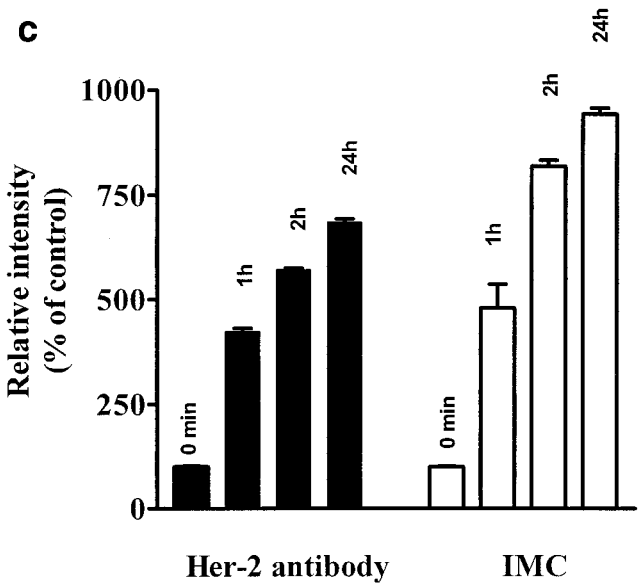

FIGURE 2 – CONTINUED.

Determination of serum calcium levels

Quantitative determination of serum calcium levels was performed by colorimetric analysis using calcium (Arsenazo III) reagent (Sigma Diagnostics, St. Louis, MO). Calcium in the serum reacts to the Arsenazo III reagent and forms purple-colored calcium-Arsenazo III complex. The intensity of the color is read at 600 nm and evaluated in relation to the color intensity obtained by processing simultaneously known calcium standards (Sigma Diagnostics, St. Louis, MO). Data represent the mean ± SE values obtained in 5 animals.

Statistical analysis

Statistical significance between groups was determined by ANOVA. Differences between group mean values were determined using Dunnet's or Bonferroni multiple comparison tests. A p-value < 0.05 was considered a statistically significant difference. Data represent the mean ± SE.

RESULTS

Characteristics of IMC

The D5-Her-2 IMC prepared with different molar concentrations of antibody to SANPAH was subjected to a 4–20% SDS-PAGE. After electrophoresis, the molecular weight of the IMC was increased with higher molar concentrations of antibody to D5. At higher molar concentrations of SANPAH, electrophoretic mobility of the antibody SANPAH was decreased. We used SANPAH at 1:40 concentration and prepared IMC with different molar concentrations of D5. However, at 1:40 antibody to SANPAH and 1:40 antibody to D5 molar concentration (6.67 μM antibody: 266.8 μM of SANPAH and D5 each), the IMC retained the ability to bind to Her-2 receptors. Thus, in all experiments, the IMC was prepared using 1:40:40 molar (Her-2 antibody:SANPAH:D5) concentration.

Competitive binding of IMC with Her-2 antibody for Her-2 receptor binding sites in BT-474 cells

As shown in Figure 1, the percent binding of both the ^{125}I Her-2 antibody and the ^{125}I IMC was reduced when the cells were incubated with increasing concentrations of the unlabeled antibody, suggesting that both the ^{125}I Her-2 antibody and the ^{125}I Her-2 antibody immunolinked to D5 compete with the unlabeled Her-2 antibody for Her-2 receptor binding sites in BT-474 cells (Fig. 1). The nonradioactive control antibody or its IMC failed to compete with the ^{125}I Her-2 antibody (data not shown).

Internalization of the IMC in BT-474 cells

We examined whether the Her-2 antibody, the isotype matched control nonspecific antibody or their IMC linked to D5 are internalized in BT-474 cells showing over expression of Her-2 receptor. At 0 min time (untreated cells), we did not observe any FITC signal in the cells. After 10 min incubation with the Her-2 antibody, intense immunostaining was observed on the cell surface; after 30–60 min incubation, immunostaining was mostly on the cell surface but a few vesicular bodies were also detected in the cytoplasm. After 2 h incubation, the cell surface staining was present but cytoplasmic vesicular staining had increased. At 24 h, numerous FITC-labeled vesicular bodies were detected in the cytoplasm. Figure 2*a* shows representative confocal images of DAPI-stained cells, FITC-labeled cells and combined pictures showing DAPI and FITC labeling obtained in BT-474 cells after 0, 10 min, 2 hr and 24 hr incubation with the Her-2 antibody. Insets in each image show cropped images of isolated cells.

We also determined the internalization of IMC. BT-474 cells were incubated with IMC for 10 min to 24 h, and then the cells were incubated with FITC-labeled secondary antibody and the localization of IMC (Her-2 covalently linked to D5) was determined under a laser scanning confocal microscope. In general, the immunostaining pattern was similar to that observed with the Her-2 antibody. In untreated (0 min) cells, no immunostaining was detected in BT-474 cells. Distinct cell surface immunostaining was observed between 10 min to 2 h. We also observed the presence of vesicular bodies in the cytoplasm after 2 h, and this was at maximum level at 24 h (Fig. 2*b*).

As experimental controls, we also simultaneously processed cells incubated with the control antibody or the IMC of control antibody. We did not observe cell surface or specific intracellular immunostaining at any time point during the course of our study (data not shown).

Quantitative analysis of time dependent accumulation of the Her-2 antibody or its IMC in BT-474 cells was performed by flow cytometry. Both Her-2 antibody and IMC showed a time dependent increase in fluorescence intensity; maximum intensity was noticed after a 24 h incubation with respective agents. Comparatively, fluorescence intensities were similar in cells incubated with Her-2 antibody and its IMC throughout the course of our study (Fig. 2*c*).

Effect of IMC on intracellular lipid accumulation in BT-474 cells

We examined the accumulation of intracellular lipid droplets as a marker of breast cell differentiation in BT-474 cells treated *in vitro* with D5, Her-2 antibody or IMC for 7 days. In all treatment groups, the cell surface was stained bright orange, whereas intracytoplasmic lipid droplets stained bright yellow. Lipid droplets were generally localized in the cytoplasm around the nuclear periphery. In vehicle-treated control cells, few small lipid droplets were noticed. On the other hand, compared to vehicle-treated control cells, we observed increased accumulation of bright yellow lipid droplets localized around the nuclear periphery in cells treated with Her-2 antibody, D5 or IMC (Fig. 3). Control antibody or IMC to control antibody showed few lipid droplets, similar to that in vehicle treated control (see inset in Fig. 3*a* and 3*d*) cells. In cells pretreated with isopropyl alcohol before Nile Red staining, lipid droplets were not observed, whereas cell surface orange staining was similar to that observed in cells stained without isopropyl alcohol treatment (data not shown).

Effect of IMC on casein accumulation in BT-474 cells

We examined accumulation of intracellular casein granules as a marker of breast cancer cell differentiation after various treatments. In control vehicle-treated cells, we did not observe casein granules in the cytoplasm. After treatment with D5, we observed

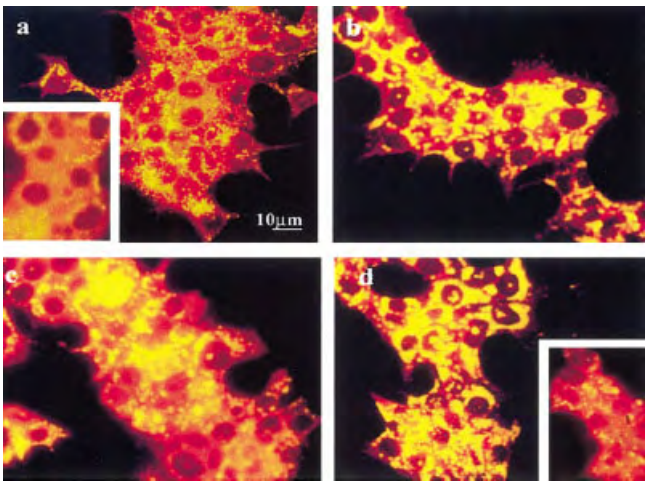

FIGURE 3 – Lipid staining in BT-474 cells incubated for 7 days in MEM-E containing 5% charcoal-treated FBS alone *(a)* or containing 5 μg/ml Her-2 antibody *(b)*,10^{-6} M 1α(OH)D5 *(c)* or IMC *(d)*. At the end of incubation, cells were washed with HEPES buffer and then stained with Nile Red stain. Bright yellow stain indicates neutral lipid, and bright orange stain is due to phospholipid staining. Insets: treated with control antibody *(a)* or control antibody IMC *(d)*.

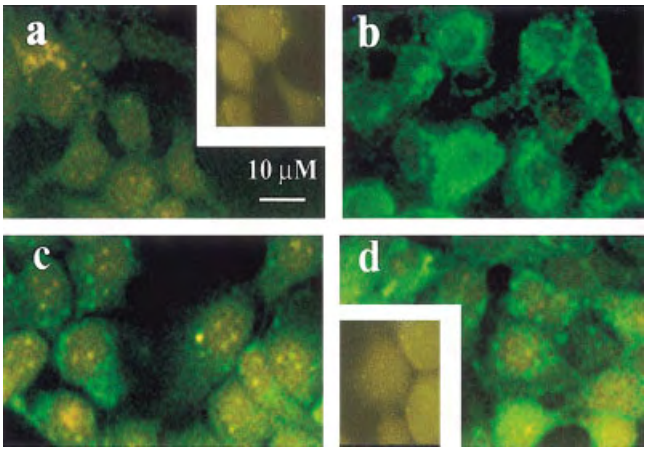

FIGURE 4 – Beta casein immunostaining in BT-474 cells incubated in culture medium containing vehicle only *(a)*, Her-2 antibody (1.5 μg/ml) *(b)*, 1 μM 1α(OH) D5 *(c)* or IMC (equivalent to 1.5 μg Her-2) *(d)*. Green fluorescent granules show casein staining. Insets: treated with control antibody *(a)* or control antibody IMC *(d)*.

presence of casein granules in the cytoplasm. Similarly, both Her-2 antibody- and IMC-treated cells exhibited more accumulation of casein granules than did vehicle-treated control cells The immunostaining pattern in IMC-treated cells was similar to that observed in D5-treated cells. In both treatments, the accumulation of large distinct granules scattered in the cytoplasm was observed. Staining in antibody-treated cells and in control antibody IMC-treated cells was similar to that observed in vehicle-treated control cells (see inset in Fig. 4*a* and 4*d*).

Effect of IMC on in vitro *proliferation of BT-474 cells*

For proliferation, Ki-67 was determined in cells incubated for 7 days with various treatment agents or a combination of Her-2 antibody and D5. Ki-67 staining (%) was similar in cells treated with vehicle, Her-2 antibody, control antibody or control antibody IMC. BT-474 cells treated with D5 and IMC exhibited significant ($p < 0.05$) reduction in % Ki-67 stained cells. These results are presented in Table I.

Effect of IMC on in vivo *growth of BT-474 cells in athymic mice*

The effect of 1α(OH) D5 supplemented in the diet was examined in female athymic mice transplanted with BT-474 cells. In animals treated with dietary D5 supplement, tumor volumes (cm^3) were significantly ($p < 0.05$) smaller than those in the control group at days 21, 28 and 47 after initiation of the treatment. Mean tumor volumes in control and Her-2 antibody treated groups were similar throughout the course of our study. In animals treated with IMC, mean tumor volumes were significantly ($p < 0.05$) smaller than in the controls or HER-2 antibody-treated group, and were similar to that in the D5-treated group at 21–47 days after initiation of treatment (Fig. 5). In a separate experiment, we also determined the effect of D5 given intraperitoneally ($n = 8$) once weekly for 4 weeks. The control group ($n = 10$) received ethanol (solvent volume similar to that in D5 preparation). Mean tumor volume in animals given intraperitoneal D5 (data not shown in Fig. 5) was similar to that in the control group (day 23, 0.13 + 0.02 *vs.* 0.13 ± 0.02; day 33, 0.30 ± 0.04 *vs.* 0.38 ± 0.05).

Serum calcium levels in animals treated with IMC

Serum calcium levels were slightly but not significantly higher in those animals treated with D5 than in vehicle-treated controls (9.37 + 0.26 *vs.* 8.41 + 0.39 mg/dl, $n = 5$ in each group). The Her-2 antibody treatment had no significant effect on calcium levels (7.99 + 0.29 mg/dl, $n = 5$ in each group). In animals treated with IMC, serum calcium levels were significantly ($p = 0.05$) lower (7.45 + 0.63 mg/dl, $n = 5$) than in those animals treated with the D5 in the diet; they were similar to the levels in the Her-2 antibody-treated group and were not significantly different from those in the control group.

DISCUSSION

We recently identified a vitamin D analog that induces differentiation-associated biomarkers in breast cancer cells; *in vivo* and *in vitro*, it inhibits growth of the selected human breast cancer cell

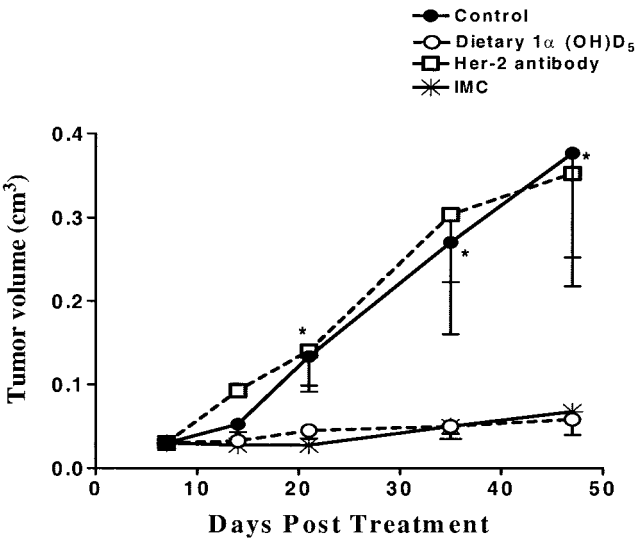

FIGURE 5 – Mean tumor volume in control vehicle-treated animals receiving dietary supplement of 1α(OH)D5 (12.5 μg/kg diet), animals treated with the Her-2 antibody (5 μg) intraperitoneally once weekly during the course of study and animals treated with IMC intraperitoneally once weekly during the course of study. Data represent the mean tumor volume ± SE in each group. *Indicates significant difference between control and D5 (dietary) and IMC treatment groups.

TABLE I – KI- 67 IMMUNOSTAINING IN BT-474 CELLS[1]

Treatment	% of cells stained
Control (vehicle only)	43.6 + 5.7 (n = 10)
1α (OH)D5 (1 μM)	10.23 + 2.3 (n = 10)[1]
Her-2 antibody (1.5 μg/ml)	45.18 + 3.8 (n = 10)
Control antibody (1.5 μg/ml)	46.9 + 2.89 (n = 10)
IMC	11.5 + 6.7 (n = 13)[1,2,3]
Control antibody immunoconjugate	42.71 + 1.67 (n = 10)

[1]The cells were incubated at 37°C in the culture medium containing 5% FBS for 7 days in the presence of vehicle, D5, Her-2 antibody, IMC, control antibody or its immunoconjugate. Number in parentheses indicates number of independent observations.– [2]Significant difference between control and treatment groups.– [3]significant difference between Her-2 antibody and IMC treated group.– [4]significant difference between IMC and control antibody immunoconjugate.

lines.[15,16] Detailed preclinical toxicity studies suggested that this vitamin D analog is well tolerated in experimental animals.[19] Although D5 is shown to be nontoxic in 28-day toxicity studies, if used continuously for a prolonged time period it could potentially develop a calcemic effect. Also, if the compound is linked to the cancer cell-specific receptor protein, it could be delivered to specific sites without affecting other normal tissues. In our study, we evaluated whether targeted delivery of D5 by linking it to a commercially available mouse monoclonal Her-2 antibody is effective in inhibiting the growth of breast cancer cells expressing high amounts of Her-2 protein. Even though it would be ideal to use herceptin (a humanized Her-2 antibody currently in use in the clinic), we used mouse monoclonal antibody, which by itself has no significant effect on the growth of breast tumor cells as a carrier protein. The latter characteristic allowed us to determine precisely whether targeted delivery of D5 to breast cancer cells has therapeutic benefit.

We confirmed that the Her-2 antibody used in our present study, when immunolinked to D5, retains its original properties and competes with intact unconjugated native antibody for the Her-2 receptor binding sites on breast cancer cells. Detailed time-course study also suggested that IMC undergoes internalization. Detailed time course analysis by confocal microscopy showed that both Her-2 antibody and IMC bind to the cell surface and then localize to the cytosol in vesicular form. Whether D5 linked to the Her-2 antibody dissociated from the IMC and then entered the nucleus is unclear. However, based on the effect of IMC on cell proliferation *in vivo* and *in vitro*, we speculate that once the IMC is internalized, the Her-2 antibody is degraded and then D5 is released in the cytosol and translocated to the nucleus.

We further confirmed that both D5 and the Her-2 antibody in the IMC retained their cell-differentiating properties. IMC is able to induce cell differentiation, as evident from the increased accumulation of intracellular lipid droplets. In our previous study, we showed that in MCF-7 and BT-474 cells, intracellular lipid accumulation is increased after D5 treatment compared to the vehicle-treated control.[15,16] We confirmed the cell-differentiating action of IMC by examining another breast cell differentiation marker: beta casein. Generally, beta casein, a major component of milk, is produced by differentiated breast cells; breast cancer cells fail to produce casein. In our study, we observed the accumulation of casein granules in BT-474 cells treated with D5, the Her-2 antibody and IMC. These results collectively indicate that D5 and the Her-2 antibody individually have cell-differentiating action on BT-474 cells, and that the latter characteristic is preserved when these 2 compounds are immunoconjugated.

In vitro, D5 showed growth-inhibitory action in BT-474 cells, as evident from the percentage of Ki-67 positive cells after 7 days treatment. Similarly, IMC treatment also exhibited growth-inhibitory action. In contrast to cell-differentiating action, Her-2 antibody did not show an effect on cell proliferation. These results are in agreement with those reported previously by Van Leeuwen and associates.[20] In that report, the effect of 9G6.10 antibody was determined in various cell types harboring normal or mutated Her-2 protein. The antibody 9G6.10 (40 μg/culture dish) showed no significant influence on colony formation in soft agar of those cells with normal Her-2 protein; however, it significantly inhibited the incidence of colony formation of cells transfected with mutant Her-2. BT-474 cells express the nonmutated Her-2 protein, and in our study the 9G6.10 Her-2 antibody had no effect on cell growth. Our results on the effects of the Her-2 antibody on cell proliferation and the expression of various differentiation markers collectively suggest that cell-differentiating and antiproliferative actions are mediated through different molecular pathways.

The effects of the D5, Her-2 antibody and its IMC were evaluated on the growth of BT-474 cells transplanted in athymic mice. When given separately by intraperitoneal injection at an equivalent concentration to that used in the IMC, vehicle treatment, Her-2 antibody treatment, and D5 each failed to inhibit *in vivo* growth of BT-474 cells. Interestingly, dietary supplement of D5 showed significant inhibition on tumor growth. These results are in agreement with our previous findings in UISO-BCA-4 breast cancer cells.[15] Furthermore, administration of IMC (once weekly) also inhibited the growth of BT-474 cells. We believe that the Her-2 antibody in IMC may simply act as a carrier protein to deliver D5 specifically to Her-2 expressing breast cancer cells. Thus, IMC is able to increase the intratumoral concentration of D5, which in turn inhibits the growth of breast cancer cells. Alternatively, D5 immmunoconjugated to Her-2 antibody is stable and is retained by the tumor cells for a prolonged period, making it effective in inhibiting the *in vivo* growth of breast cancer cells. The results on how different administration routes of D5 (intraperitoneal *vs*. oral in the diet) affect BT-474 cell growth suggest that when D5 is administered unconjugated, it is retained by the cells for a shorter time and thus fails to show growth inhibition. Even though the relative weekly cumulative doses of D5 were similar (whether animals received it once a week by intraperitoneal injection or received it as a dietary supplement), the growth inhibitory action on BT-474 cells was seen only in the dietary group. These results suggest that D5 needs to be given frequently to achieve and maintain the intratumoral concentration necessary for tumor growth inhibition. Generally, a steady blood level of the compound is maintained when given continuously in the diet, and that in turn may help to maintain a sustained concentration of D5 available to tumor cells. It is possible that administration of D5 by the intraperitoneal route could rapidly generate a very high concentration (in the blood and tumor cells) that is nonetheless transient due to its metabolic clearance and which therefore fails to generate the D5 concentration (in the blood and tumor cells) necessary to inhibit the growth of BT-474 cells. Similarly, Her-2 antibody treatment alone failed to show growth inhibition, and even though it showed potent cell differentiation in *in vitro* assay, we also postulate that the cell-differentiating effect of the Her-2 antibody is probably transient and thus fails to inhibit cell proliferation *in vivo*.

The effect of IMC on *in vivo* tumor growth was similar to that observed in animals receiving a daily supplement of D5. However, it appears that IMC therapy is relatively safer than the dietary supplementation. *In vivo*, dietary supplement of D5 slightly increased serum calcium levels; however, IMC treatment did not show significant increase in serum calcium levels. Recently, vitamins (especially active metabolites of vitamin D, 1,25(OH)2D3, and its synthetic analogs) are known to have potent growth inhibitory and cell differentiating actions in various cancer cell types, including breast cancer. However, the use of vitamin D-related compounds for the treatment of breast cancer is hindered due to their hypercalcemic activity. Results from our study suggest that it is feasible to use known vitamin D metabolites or analogs with potent growth inhibitory or cell differentiating actions as a therapeutic agent, even though they are toxic, using the IMC approach described in our present study. Also, even though the Her-2 antibody used in our study has no direct clinical application, the concept of the IMC used in our study has potential therapeutic value. We believe that tumor cells positive for Her-2 receptor but

refractive to the growth-inhibitory effect of Her-2 antibody therapy could be targeted by D5-like vitamin D analogs using an IMC approach. In conclusion, the results from our present study identify a possible new, safe tumor cell-targeted therapy for the treatment of highly aggressive Her-2-overexpressing breast cancer. Further studies are in progress using herceptin as a D5 carrier protein to determine whether D5 immunolinked to herceptin is more effective at inhibiting breast tumor growth and metastasis than herceptin treatment alone.

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Vitamin D and Cancer Chemoprevention

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Abstract

It is now well recognized that the active metabolite of vitamin D plays a significant role as a modulator of cell proliferation and differentiation in addition to its principal function in bone mineralization. However its clinical use in cancer prevention and therapy is hampered due to its toxic effects at efficacious concentration. Therefore, there is a considerable effort diverted towards designing and synthesizing chemical analogs of vitamin D that are less hypercalcemic and more potent in inhibiting growth of cancer cells. The chemopreventive efficacy of a few analogs of vitamin D has been evaluated in skin, prostate, colon and mammary carcinogenesis. Results have shown that in mammary epithelial cells 1α-hydroxyvitamin D5 is selectively active against the carcinogen-transformed cells. Other investigators have shown that 1α,25 (OH)2D3 provides antiproliferative activity in normal breast epithelial cells also. In mouse mammary gland organ cultures, 1,25(OH)2D3, EB1089, Ro24-5531 and 1α(OH)D5 suppressed development of carcinogen-induced preneoplastic mammary lesions. In chemically induced mammary carcinogenesis models several analogs including EB1089, 1α(OH)D3,1α(OH)D5, Ro24-5531 and CB1093 have shown protective efficacy. In colon the effects of Ro24-5531, 1α(OH)D5, against carcinogen-induced aberrant crypt foci and efficacy of 1,25,(OH)2D3-16-ene-19-nor-24-0xo-D3 in APC(min) mice on tumor burden have been reported. The genomic action of VDR is mediated by nuclear vitamin D receptors. VDR knockout mice develop phenotypically abnormal glands and respond to carcinogen more aggressively. Results in the literature support that the VDR mediated action is brought about by the modulation of the target organ specific signaling pathways. Several vitamin D analogs are being evaluated clinically and developed for possible chemoprevention or progression of the disease.

Key words: Vitamin D, mammary carcinogenesis, chemoprevention, and Vitamin D receptors

Chemoprevention

The term cancer chemoprevention was coined by Michael Sporn approximately 28 years ago (1) to encompass the, suppression of carcinogenesis by means of chemopreventive

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agents which are natural or synthetic chemicals that block, arrest or reverse the development and growth of tumors. The process of carcinogenesis can be divided into three identifiable stages: initiation, promotion, and progression (2) The process of initiation can be defined as the transformation of a normal cell into an initiated cell in response to carcinogenic or mutagenic stimuli. The process of initiation can often be blocked by anti-initiating chemicals such as antioxidants (3). Not all initiated cells form tumors; however, in response to exogenous and endogenous growth promoting factors, initiated cells can progress to a preneoplastic stage which is often recognized as a promotion stage. Agents that can affect the growth arrest of initiated cells are considered antipromotional agents. During the past two decades this field has identified numerous classes of compounds that can be classified as antipromotional chemopreventive agents. These classes include: retinoids, inhibitors of polyamine biosynthesis, inhibitors of cyclooxygenase-2, inhibitors of calcium channel blockers, lignans, flavonoids, antihormones, phytoestrogens such as genistein, inhibitors of farnesyl transferase, and modulators of Peroxisome Prolifaerator Activated Receptor (PPAR) and deltanoids (vitamin D analogs) (4, 5). Although the mechanisms of action are specific for each class of agent, ideally they all suppress the proliferation of transformed cells into a putative preneoplastic lesion by arresting cell division through either the induction of programmed cell death or cell differentiation at non-toxic concentrations. A preneoplastic lesion progresses to develop cancer. The stages of carcinogenesis and their possible modulation by chemopreventive agents are shown in a diagram in Figure 1. Once cancer has developed, treatment with more toxic drugs becomes acceptable because the risk to benefit ratio then favors the benefit from toxic drugs, whereas such toxicity is unacceptable for a chemopreventive agent. Although the stages of carcinogenesis have been well defined and logically displayed, there is uncertainty as to where the prevention ends and therapy begins. In recent years many chemopreventive agents have been evaluated for their efficacy during disease progression using xenografts models, which are traditionally considered chemotherapy models. The concept of chemoprevention has evolved over the years. It has become clear that, in addition to mediating a phenotypic response to chemopreventive agents, they must modulate a genomic response [6, 7]. This would, in turn, modify signal transduction processes within the cell to acquire or maintain normal cell function. Thus, chemopreventive agents can be defined as chemicals or combinations of chemicals that can alter or reverse the expression or function of molecular targets responsible either for the transformation of normal cells or for supporting the proliferation of transformed cells that become cancer.

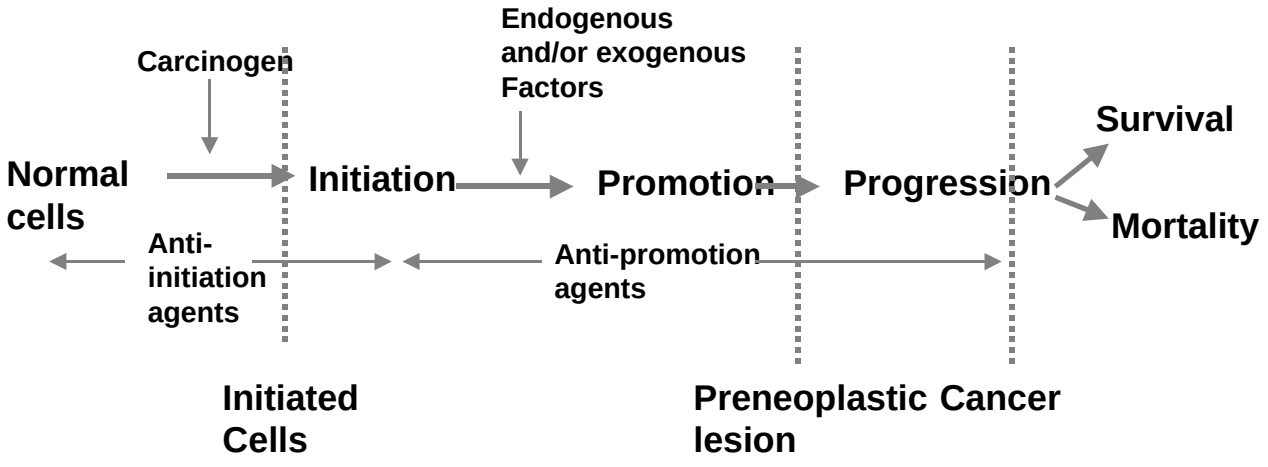

Figure 1. Schematic diagram to show stages in mammary carcinogenesis and efficacy of chemopreventive agents

Vitamin D

Vitamin D is steroid hormone from a family of 9,10 secosteroids and differs in its side chain structures. It is classified into two major classes: ergocalciferol (Vitamin D2) and cholecalciferol (vitamin D3) and further divided into D4, D5, D6 and D7. The form vitamin D6 is a 24-ethyl-ergocalciferol, whereas vitamin D5 is a 24-ethyl-cholecalciferol. Vitamin D4 and D7 are 24 methyl-cholecalciferol either in the *trans* form (D4) or the *cis* form (D7) (8). Toxicity rating indicated that, of all the forms of Vitamin D, D3 is the most toxic and D5 the least toxic (Figure 2) (9). Vitamin D is obtained from the diet or derived from 7-dehydrocholesterol upon exposure of the skin to sunlight. Interaction of 7-dehydrocholesterol with UV light results in photolysis and cleavage of the B-ring of the steroid molecule, which after thermo-isomerization becomes a secosteroid. The pro-hormone gets metabolized in the liver to 25-hydroxyvitamin D3 and then in the kidney by 1α-hydroxylase to 1,25(OH)2D3, the active metabolite and ligand for the vitamin D receptor (VDR) (10). The principal role for 1,25(OH)2D3 is the calcitropic effect of increasing calcium uptake in the intestine for the regulation of bone calcification (11). In addition to this primary regulation of calcium homeostasis, the active metabolite of vitamin D is known to have a potent effect on the induction of cell differentiation in leukemia cells and other cancer cells of epithelial origin (12). This suggests that vitamin D could be of significant clinical importance in the prevention and treatment of cancer in several target organs. In recent years it has come to light that, in addition to kidney cells, epithelial cells which respond to vitamin D include breast, prostate and colon epithelial cells also produce 1α-hydroxylase. Therefore these vitamin D target cells can further metabolize 25-hydroxyvitamin D3 to the active metabolite at the site of action (13, 14). These global routes of metabolism and potential application of vitamin D are summarized in Figure 3.

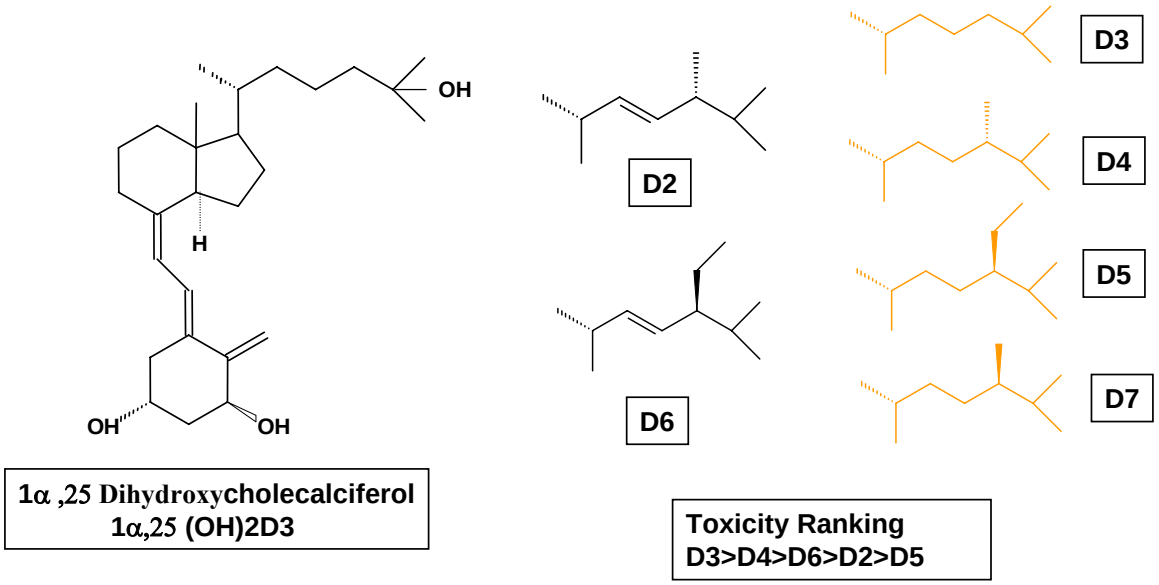

Figure 2. Structural side-chain differences in vitamin D

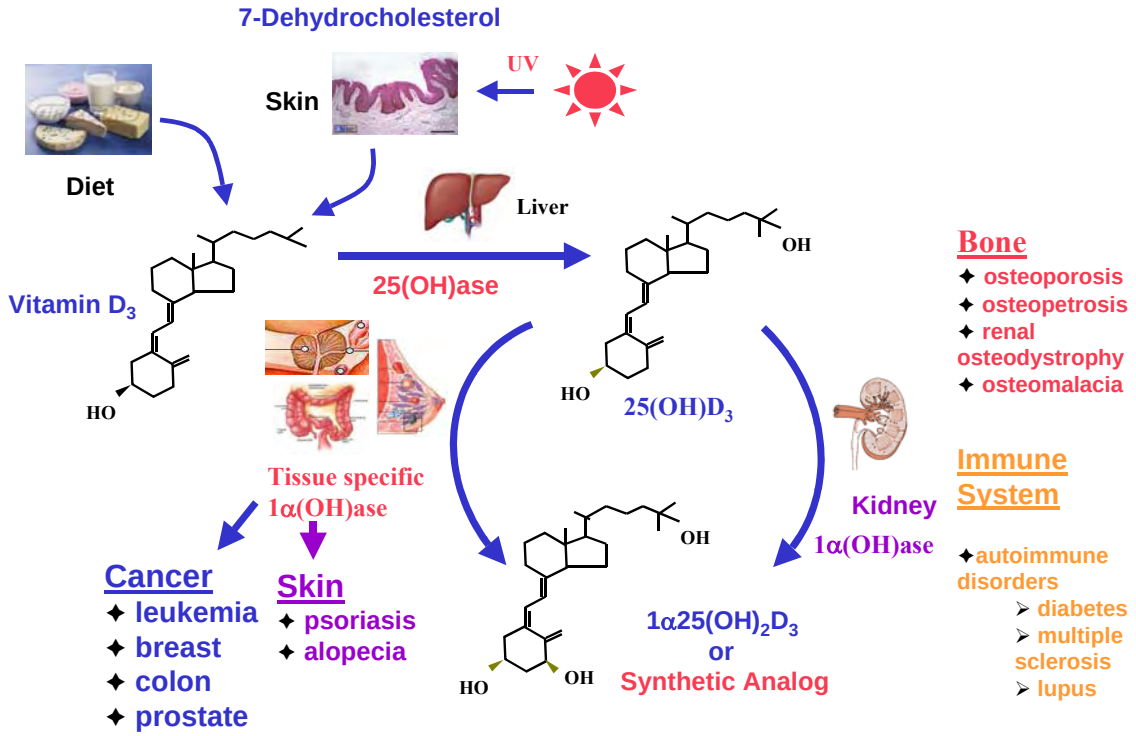

Figure 3. Potential Applications of Vitamin D Analogs

Vitamin D Analogs

The significance of 1,25(OH)2D3 as a potent inducer of cell differentiation was first recognized in myeloid leukemia cells (15). Since this early report, numerous reports in many cell types have confirmed its effects on cell differentiation and on cell proliferation (16, 17). Yet its clinical use has been hampered by its adverse toxic effects. At efficacious concentration, 1,25(OH)2D3 induces severe hypercalcemia (18). This limitation has prompted many investigators to design and synthesize analogs of vitamin D with hope of reducing its toxicity and enhancing efficacy. More than 1000 analogs of vitamin D have been synthesized and evaluated in selective experimental models. Although alteration in the vitamin D molecule can be made on the rings as well as on the open side chain, maximum modifications have been made on the side chain (19, 20). Despite the synthesis of hundreds of analogs, only a handful have progressed to efficacy evaluations in in vivo experimental models, or to further mechanistic experiments leading towards clinical trials. In addition to the natural metabolite 1,25(OH)2D3, some of the more prominent analogs include (but are not limited to): EB1089 (Seocalcitol); 22-oxa-1α,25(OH)2D3 (OCT, Maxacalcitol); 16-ene analogs such as 1,25-dihydroxy-16-ene-23-yne-26,27-hexafluorocheolecalciferol (RO24-5531); 24R,25-dihydroxycholecalciferol; calcipotriol (MC 903); and 1α-Hydroxy-24-ethyl-cholecalciferol (1α(OH)D5). In addition, new analogs are continually being designed and synthesized in order to obtain higher activity and reduced toxicity. More recently, the effects of 19-nor-1α,25(OH)2D2 (zemplar) and 21-(3-methyl-3-hydroxy-bytyl)-19-nor-cholecalciferol (Gemini-19-nor, an analog with two side chains) have been intensely studied for therapeutic and preventive efficacy in prostate and leukemia cells (21, 22).

Cancer Chemoprevention by Vitamin D Analogs

The efficacy of chemopreventive agents, including analogs of vitamin D, are largely evaluated using established human cancer cell lines as models for specific target organs. Chemopreventive activity, in principle, however, should be examined during cell transformation and during disease progression of the

transformed cells in experimental conditions. These studies are often difficult due to the unavailability of selective models. Therefore, the chemopreventive effects of vitamin D analogs are largely evaluated only for breast, colon, skin, and prostate cancer.

Chemoprevention in Cell and Organ Culture Models

The effects of vitamin D analogs on normal cells or in cell transformation have not been extensively studied. Early studies evaluated the effects of 1,25(OH)2D3 on normal and leukemic myeloid clonogenic cells. Results showed that mature myeloid leukemic cells responded to vitamin D and exhibited induction of cell differentiation and inhibition of clonal cell growth, whereas the D-analogs stimulated the growth of normal cells (23). Moreover leukemic cells from patients were inhibited by 1,25(OH)2D3 while normal granulocyte-monocyte-stem cells were not. These results indicate that the effects of vitamin D analogs might be selective for transformed cells. On the other hand, experiments using human keratinocytes more recently showed that 1,25(OH)2D3 and other analogs such as calcipotriol, tacalcitol, and maxacalcitol suppressed keratinocyte proliferation and induced differentiation (24). In addition, the role of vitamin D analogs has been extensively investigated in normal and transformed breast epithelial cells. In a study to examine the role of TGFβ-signaling in vitamin D action, Yang et al. (25) showed that nonmalignant MCF10A and 184A1 cells were sensitive to 1,25(OH)2D3, whereas late passage MCF7L cells were not responsive to D3. Transfection of MCF7L cells with TGFβRII transcripts were sensitive to EB1089 and to 1,25(OH)2D3. More recently, Welsh and colleagues observed antiproliferative effects of 1,25(OH)2D3 in normal mammary epithelial cells (26). In contrast to those results, Gache et al. (27) showed that whereas MCF7 and tumor cells derived from breast cancers responded to EB1089 and 1,25(OH)2D3, normal breast epithelial cells did not exhibit an antiproliferative response to vitamin D analogs. Our results are similar to the later report. We transformed the normal breast epithelial cells MCF12F with N-mrthyl-N-nitrosourea (MNU) or 7,12-dimethylbenz(a)anthracene (DMBA) and generated the transformed cell lines $MCF12F_{MNU}$ and $MCF12F_{DMBA}$. These cell lines have altered growth characteristics, and 1α(OH)D5 inhibited the proliferation of the transformed cells but not the normal cells. The cells were arrested in the G1 phase of the cell cycle. This was accompanied by the induction of VDR. A comparative gene array profile indicated that, between normal and cancer cell mRNA, nearly 300 genes were differentially expressed; some of the selective genes were reversed in their expression after incubation with 1α(OH)D5. These results suggest that the effects of this analog of vitamin D might be selective for transformed or cancer cells (28). A summary of response to vitamin D analogs for various cancer cell lines is shown in Table 1.

The effects of chemopreventive agents can be determined in organ cultures. Organ culture models for prostate, skin, colon and mammary glands have been employed. However, only the mammary gland organ culture model has been extensively exploited for chemoprevention research. In this model, mammary glands from young mice respond to growth promoting hormones and structurally differentiate into alveolar structures resembling glands during pregnancy. Removal of hormones from the culture makes the glands regress back to the ductal stage. During the growth phase, the glands, if exposed to a carcinogen, develop precancerous unregressable lesions, which can be prevented if the glands are incubated with chemopreventive agents (29). We have evaluated more than 200 chemopreventive agents using this model and there appears to be a high degree of correlation with the agent identified in this model with that of in vivo carcinogenesis. Using this organ culture model we showed that 1,25(OH)2D3, EB1089, RO24-5531 and 1α(OH)D5 suppressed the development of DMBA-induced preneoplastic lesions (30). By dissecting the time of exposure of 1α(OH)D5 during the culture, we showed its effect to be more prominent during the promotion phase of carcinogenesis. The vitamin D analogs induced VDR and TGFβ in the mammary glands, indicating that the action of 1α(OH)D5 may be mediated by VDR. Normal mammary glands in the absence of DMBA did not respond to 1,25(OH)2D3 or 1α(OH)D5, indicating that the response was selective for the transformed cells (31). Similar results were also obtained with human breast tissues obtained during

surgery. Incubation with 1μM 1α(OH)D5 did not have any effects on the morphology of the normal epithelial cells but the tumor cells underwent extensive apoptosis.

Chemoprevention in Animal Models

The efficacy of a chemopreventive agent in the process of cancer development can be best studied using experimental animal models. Over the past 20 years, target organ specific models have been developed and widely used (32). The development of a carcinogenesis model often involved refining the selection of a carcinogen that will induce tumors only in the target organ under evaluation. These models include: DMBA-induced and TPA-promoted skin cancer; DMBA or MNU induced mammary cancer; MNU-induced rat prostate cancer; Azoxymethane or dimethylhydrazine (DMH)-induced colon aberrant crypt foci or tumors; diethylnitrosoamine (DEN) or aflatoxin-induced liver cancer; N-butyl-butyl-nitrosamine (OH-BBN)-induced urinary bladder cancer; and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)- induced lung cancer. In addition, in recent years several transgenic and gene-knockout rodent models have been developed to help understand the molecular mechanisms of the carcinogenesis process and of chemopreventive agents. Although there is considerable evidence in the literature regarding possible effects of vitamin D analogs in cell culture models, there is a very limited number of reports available on the chemopreventive efficacy of vitamin D analogs in vivo. Experimental evidence has been reported for colon, skin, prostate and mammary carcinogenesis, and the results are summarized in Table 2. Since the primary aim of chemoprevention study is to observe the non-toxic nature of the chemopreventive agent, for all potential preventive agents the primary requirement is to identify a non-toxic maximum tolerated dose in the experiments. For vitamin D analogs the dose has to be non-hypercalcemic and must not affect body weight or induce kidney damage. All vitamin D analogs evaluated for chemopreventive activity have been used at a concentration that did not induce hypercalcemia (33). Yet in many studies, at the end of the experiment, hypercalcemia was noted.

Table 1. Summary of efficacy of vitamin D analogs in cancer cell proliferation

Target organ	Cells	Vitamin D analogs	Efficacy	Comments
Breast	ER+			
	MCF-7, ZR75-1, T47-D	22-oxa-calcitriol, 1α(OH)D_5,EB-1089,	All effective	VDR+
	BT474, BT20, SK-BR-3	KH1060, MC903, RO24-5531, 22-oxa-Calcitriol		
	ER-			
	MDA-MB-231, MDA-MB-436	1α-(OH)D_5, 22-oxa-calcitriol, KH1060, RO24-5531	Ineffective	VDR+/-
	UISO-BCA-4	1α(OH)D_5	Effective	VDR+
	UISO-BCA-1	1α(OH)D_5	Ineffective	VDR-
	MDA-MB-231,	22-oxa-calcitriol	Effective	VDR+/-
Prostate	LnCap, PC-3	1α(OH)D_5, EB1089, RO24-2637, 22-oxa-calcitriol, MC903	All Effective	VDR+
	Du-145	1,25$(OH)_2D_3$, RO23-7553	Ineffective	VDR+/-
	Du-145	RO24-5531, RO26-2198	Effective	
Colon	HT-29, CaCo-2	1,25$(OH)_2D_3$, RO24-5531	Effective	VDR+

Table 2. Summary of efficacy of vitamin D analogs in cancer cell proliferation

Organ	Model	Analog	Efficacy	Comments
Breast	Normal cells	$1,25(OH)_2D_3$	Antiproliferative/No effect	Both activities are reported
	VDR-KO	$1,25(OH)_2D_3$, EB1089	No response	
	Transformed	1α-Hydroxyvitamion D_5	Effective	Effect selective for transformed cells
	MNU-induced adenocarcinoma	RO24-5531, ,	Effective	No toxicity
		1α-Hydroxyvitamion D_5	Effective	No hypercalcemia
			Dose related effect	No loss of body weight
		1α-hydroxy D_3	growth inhibition	Treatment schedule
		$1,25(OH)_2D_3$	Effective	Hypercalcemia
		MC903	Effective	Hypercalcemia
		EB1089	Effective	Hypercalcemia
	DMBA-induced	1α-Hydroxyvitamin D_5	Effective (Promotion)	No toxicity
	VDR-KO + DMBA	$1,25(OH)_2D_3$	More aggressive growth	Protective effect of VDR
Skin	VDR-KO + DMBA	None	Skin papilloma	Protective effect of VDR
	APC (min) mice	1,25(OH)2D3, 16-ene-19-nor-24-oxo dihydroxy D3		
	2-stage carcinogenesis	24 or 25-t-butyl sulfone,	Effective	No Toxicity
		1-hydroxymethyl hybrid analog of D3	Effective	No Toxicity
Prostate	MNU-induced	RO24-5531	Effective	No toxicity
Colon	AOM-induced	RO24-5531	Effective	No Toxicity
	DMH-induced	22-oxa-Calcitriol, 24R,25(OH)2D3	Effective	Reduced aberrant crypt foci
	AOM-induced	1(OH)D5	Effective	Reduced ACF
	APC mice	1α-,$25(OH)_2D_3$	Effective	Effective against tumor burden,
		1,25(OH)2D3-16-ene-19-nor-24-oxo-D3	Effective	Hypercalcemic at higher conc.

The effects of several new vitamin D analogs were evaluated in a two-stage skin carcinogenesis model. Results showed that non-calcemic analogs of calcitriol -- incorporating not only 1β-hydroxymethyl alteration but also alteration of the C and D rings by introducing 24-24-fluorination or 25-t-butyl sulfones -- reduced tumor incidence as well as multiplicity of skin papilloma (34). The role of vitamin D analogs on prostate carcinogenesis has been evaluated for RO24-5531 (1.25 or 2.5 nmole/kg diet) in Lobund-wistar rats. The results of this study showed modest chemopreventive activity by RO24-5531 accompanied by no hypercalcemia, although body weight was reduced. No other chemoprevention study for prostate carcinogenesis has been reported (35). The efficacy of vitamin D analogs has been investigated in colon carcinogenesis models. The chemoprevention models have included induction of aberrant crypt foci in response to azoxymethane in mice and rats and colon tumor development. In one study dietary supplementation of 2.5nmole/kg RO24-5531 reduced the colonic tumors induced by AOM by 70% (36). No increase in calcium or phosphorous was noted. Similarly, 22-oxa-calcitriol at a high concentration of 30μg/kg i.p. was reported to reduce dimethylhydrazine induced aberrant crypts and colonic tumors in rats. However, these results were accompanied by reduced body weight (37, 38). In our

laboratory, we have recently evaluated the effects of 1α(OH)D5 at 25μg/kg diet on AOM induced ACF in CF-1 mice. Results showed that the vitamin D analog reduced ACF by 80%. There was no reduction in body weights, and the dose was not hypercalcemic. This chemopreventive effect of 1α(OH)D5 was accompanied by increased VDR, increased β-cadherin and reduced PPARδ expressions (Murillo and Mehta unpublished). These results clearly suggest a possibility of evaluating 1α(OH)D5 for colon carcinogenesis and colon cancer prevention. More recently, the effects of 1α,25(OH)2-16-ene-19-nor-24-oxo-D3 were evaluated on colon tumor development in APC mice. Results showed that the analog was efficacious in reducing tumor burden in the mice without hypercalcemia (39).

The chemopreventive efficacy of several vitamin D analogs has been reported in both MNU and DMBA-induced mammary carcinogenesis. Early studies with the natural metabolite of vitamin D at 3-nmole/kg body weights did not show any protection against MNU-induced mammary carcinogenesis On the other hand, EB1089 at 1-5 nmole/kg concentration was effective in suppressing tumor multiplicity. Similarly MC903, 22-Oxa-calcitriol, 1α(OH)D3 and CB1093 have also shown some chemopreventive effects against mammary carcinogenesis (40, 41). However, in all cases, at the end of the study toxicity was noted. An in-depth study was carried out using a non-calcemic analog, RO24-5531 in an MNU-induced mammary carcinogenesis model. It was effective at the low concentration of 2.5nmole/kg diet, when the animals were treated with a low carcinogen dose for tumor induction (42). However, this effect against tumor incidence was not observed at a high carcinogen dose level. Experiments from our laboratory showed that 1α(OH)D5 could be tolerated by rats at 116nmole/kg diet (50μg/kg diet). In older animals, at this non-toxic dose, there was a reduction in both the incidence and multiplicity of mammary tumors (43). This study was repeated with DMBA as the carcinogen in younger animals and, again, at 40μg/kg diet 1α(OH)D5 significantly suppressed tumor incidence in rats. However the tumor latency was not affected. No adverse effect on body weight or calcium levels was observed (Mehta unpublished). These results collectively suggest that vitamin D could be effectively developed as a clinical chemopreventive agent for cancers of several target organs.

Mechanism of Action of Vitamin D

To encompass the mechanism of action of vitamin D analogs in detail is not within the scope of this report. There is considerable evidence suggesting that the genomic action of vitamin D is mediated by VDR (44). As recently shown by Welsh and colleagues, VDR knockout mice exhibited an accelerated proliferation of the glands during the physiological differentiation of the glands when compared to wild type mice (45). The cells derived from VDR-KO mice were unresponsive to 1,25(OH)2D3 in culture. In addition to abnormal mammary gland development, these mice respond to DMBA more aggressively. Eighty five percent of mice also developed skin tumors in response to DMBA in 60 days in VDR-KO mice as compared to no tumors in WT mice (46). These results suggest that either lack of or altered VDR signaling may lead to hyperplasia or tumorigenesis. It has also been directly or indirectly noted that almost all efficacious analogs evaluated in chemoprevention protocols mediate their action by interacting with VDR. Studies from our laboratory showed that treatment with 1α(OH)D5 induced VDR in mammary epithelial cells. In estrogen receptor positive breast cancer cells, 1α(OH)D5 down-regulates estrogen receptors and estrogen inducible genes such as progesterone receptors and ps2, but it up-regulates the expression of VDR (28). Previously we also showed that in breast cancer cells, 1,25(OH)2D3 and 1α(OH)D5 transactivate VDRE in the presence of VDR, and that the effect was enhanced when the cells were cotransfected with RXR (47). The VDR mediated signaling of events in different target organs will probably be different. There is clear target organ specificity in the response to several chemopreventive agents (32), and this may be true for the vitamin D analogs. For example, in steroid receptor positive breast cancer, its function may be mediated by altering estrogen inducible functions; it may alter the androgen-mediated signaling pathway in prostate cancer, whereas in colon carcinogenesis it may

prevent α-catenin mediated events or modulate *Wnt*-signaling pathway (Unpublished). Thus, understanding of the mechanism of action of vitamin D and its analogs is far from complete. The differences in molecular targets in these organs are diagrammatically shown in Figure 4.

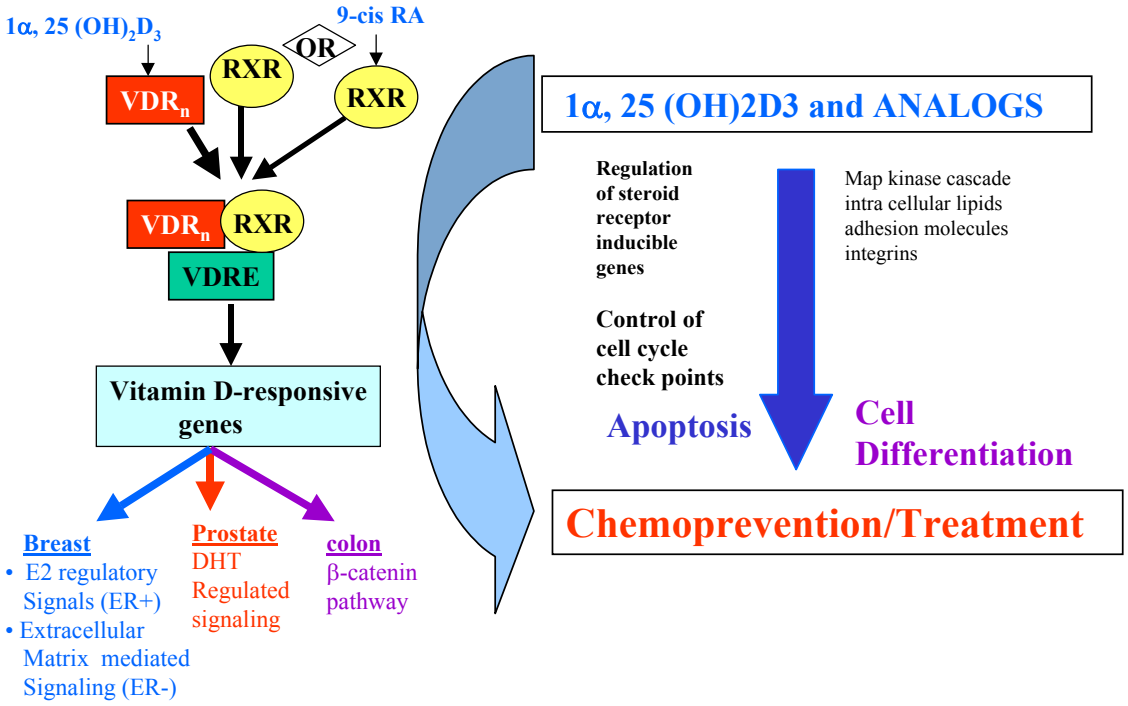

Figure 4. Possible Mechanism of vitamin D action

Future Directions

Clearly, understanding the target organ dependent molecular events that occur as a result of vitamin D analog action will provide significant insight into the discovery of a molecular target for VDR action and help with the identification of better analogs of vitamin D for target organ specific chemoprevention. Secondly, advancements on the clinical trials with selective analogs of vitamin D will provide a niche for this class of compounds and justify their use either in chemoprevention or in an adjunctive fashion for individuals with a high risk of recurrence of cancer. Finally, studies involving combination chemoprevention with vitamin D analogs and other classes of chemopreventive agents may identify a unique combination that can be used for cancer chemoprevention or for the prevention of the progression of cancer.

Acknowledgements

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Stage-specific inhibition of mammary carcinogenesis by 1α-hydroxyvitamin D5

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Abstract

Active metabolites of vitamin D are well recognised as cancer chemopreventive and chemotherapeutic agents. However, they are toxic at effective concentrations. Earlier, we reported that a non-toxic analogue of vitamin D, 1α-hydroxyvitamin D5(1α(OH)D5), inhibited carcinogen-induced mammary lesion formation in mouse mammary organ cultures (MMOC) and in *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary carcinogenesis. In the present study, we determined if 1α (OH)D5 action is selective during the initiation or promotion phases in MMOC and *in vivo*. In MMOC, 1 μM 1α (OH)D5 suppressed both ovarian hormone-dependent and -independent mammary lesions by more than 60%. Inhibition of alveolar lesions was observed only during the promotion stage ($p = 0.0016$). In a 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis experiment, 1α (OH)D5 (40 μg/kg diet) inhibited cancer incidence by 37.5% ($p < 0.05$) if 1α (OH)D5 was present in food during the promotion phase (+1 to end). However, a D5-supplemented diet during the initiation phase (−2 to +1 week) did not provide any protection. These results clearly show, for the first time, that the effects of vitamin D may be mediated selectively during the promotion or progression phases of carcinogenesis.

Keywords: Vitamin D analogue; D5; Mammary carcinogenesis; Chemoprevention; Organ culture; Preneoplastic lesions

1. Introduction

Vitamin D is a secosteroid and is classified into five major classes: ergosterol (D2), cholecalciferol (D3), 22,23 dihydroergocalciferol (D4), sitosterol (D5) and stigmasteroid (D6). The active form of vitamin D, 1,25(OH)2D3, is derived by the metabolic hydroxylation of cholecalciferol (D3) [1,2]. Toxicity studies have shown that the natural metabolite of vitamin D3 induces hypercalcaemia in animals at concentrations that provide protection against cancer formation or progression. This has led to the syntheses of analogues of vitamin D with the intention of retaining or enhancing the efficacy of vitamin D activity while reducing or eliminating its associated toxicity. More than 1000 analogues have been synthesised by various groups by modifying the side chain of the molecule, as well as introducing changes in the A and B rings. Changes in the C and D rings are not very common due to the rigidity of the structure [3,4]. Although many of these analogues have been evaluated in cell culture models for their antiproliferative activity, only a few have shown reduced toxicity and increased efficacy in *in vivo* mammary carcinogenesis models. These analogues include EB1089, KH1060, calcipotriol, RO24–5531, 22-oxa-calcitriol and 1α-24-ethyl-cholecalciferol (1α (OH)D5) [5–7]. More recently, another class of vitamin D analogues, 1α,25(OH)2-vitamin D3, with two side chains also termed as Gemini compounds, have received considerable attention since they are very active at very low concentrations [8],

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although no *in vivo* chemoprevention studies have been reported.

Cancer chemoprevention has traditionally been considered as a process that suppresses the initiation of cancer development or delays its onset [9,10]. However, there is no clear separation to suggest where prevention ends and therapy begins. It has always been generalised that chemopreventive agents are effective at non-toxic concentrations, whereas chemotherapeutic agents are often toxic. In recent years, chemopreventive agents have been evaluated both as chemopreventive agents and as possibly non-toxic chemotherapeutic agents [11]. Vitamin D analogues, like other chemopreventive agents, have been evaluated in both these settings. Results have shown that vitamin D analogues can only inhibit the growth of cells with vitamin D receptors (VDR+), indicating that the action of vitamin D is mediated by nuclear VDRs [12–14]. It has been reported that the effects of vitamin D analogues are brought about by affecting the VDR that mediates signalling, which results in a suppression of growth accompanied by either apoptosis or cell differentiation [15,12,16].

Chemoprevention can be ideally studied by inducing transformation of mammary epithelial cells and by evaluating whether or not the potential chemopreventive agent would inhibit such transformation. This has been carried out in cell cultures by transforming normal mammary epithelial cells by either Simian Virus 40(SV40) or carcinogen [17]. In organ cultures, this can be achieved by inducing transformation of mammary structures by carcinogens [18,19]. The suppression of 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced precancerous lesions in mouse mammary gland organ cultures (MMOC) has been extensively used as a model for evaluating potential chemopreventive agents [20,21]. In most cases, results have shown a correlation between chemopreventive agents efficacious in this model and their *in vivo* response [18]. We previously reported that 1α (OH)D5 suppressed mammary alveolar lesions (MAL) induced by DMBA by >60% [22]. However, whether it acts selectively against the initiation or the promotion stage of lesion formation is not known. In addition, previous *in vivo* studies showed an inhibition of carcinogen-induced mammary tumorigenesis by 1,25(OH)2D3, EB1089, RO24–5531, KH 1060 and 1α (OH)D5 at non-toxic concentrations [5,6]. However, the requirements for each analogue vary considerably. The maximum tolerated doses (MTDs) correlate well with their efficacy in carcinogenesis models. For example, in relation to 2.9 nmoles/kg 1,25(OH)2D3, the MTDs for EB1089 and RO24–5531 are 5 and 10 nmoles/kg, respectively. In comparison, 1α (OH)D5 can be tolerated at more than 100 nmoles/kg diet (42.8 μg/kg diet), without any systemic toxicity and hypercalcaemia. In the *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis model, 1α (OH)D5 inhibited the incidence of mammary tumour development in Sprague Dawley rats [23]. However, it has not been examined whether there is a selective suppression of mammary carcinogenesis during either the initiation or promotion phase. There are two animal models for experimental mammary carcinogenesis which are the most widely used [24]. Adenocarcinomas are induced in rats either by MNU or by DMBA. MNU is a direct acting carcinogen, whereas DMBA needs to be metabolised to an active carcinogen species. Both carcinogens are tissue-specific and do not induce tumours at other sites. The tumours histopathologically resemble human cancers and respond to hormonal manipulations. Since DMBA has to be metabolised to be active, one can differentiate whether the chemopreventive agent is selectively active during the initiation phase of carcinogen activation or during the promotion phase, i.e. after the cells have been transformed. In this report, we describe the stage-specific efficacy of 1α (OH)D5 in MMOC and in DMBA-induced mammary carcinogenesis *in vivo*.

2. Materials and methods

Two separate models were used to evaluate the stage-specific chemopreventive activity of 1α (OH)D5 against mammary carcinogenesis.

2.1. Mouse mammary organ cultures

There are two protocols to induce precancerous mammary lesions in the mammary glands of immature mice. The glands can either develop MAL or mammary ductal lesions (MDL), depending upon the steroid hormone combination present in the medium. If the glands are incubated in the presence of oestradiol 17*β* and progesterone, then they develop MDL [25], whereas if oestrogen and progesterone are replaced with glucocorticoids, then MAL are formed [18,20].

The procedure for the induction of mammary lesions has previously been described in detail in [18,21,22]. Briefly, thoracic pairs of mammary glands from Balb/c mice pre-treated with 1 μg oestradiol and 1 mg progesterone for 9 days were dissected free of muscles and explanted in culture dishes containing serum-free Waymouth's medium 752MB/1 supplemented with 5 μg insulin, 5 μg prolactin, 1 μg aldosterone and 1 μg hydrocortisone per ml of medium (for MAL) and incubated for 10 days. For MDL instead of aldosterone and hydrocortisone, the medium contained 0.001 μg oestradiol 17*β* and 1 μg progesterone. In order to induce the development of precancerous lesions, the glands are incubated with 2 μg/ml DMBA for 24 h on day 3 of the culture. This 10 day growth proliferative phase allows the glands to undergo structural differentiation and they appear similar to those from pregnant mice.

After 10 days, the glands are transferred to a medium containing insulin (5 μg/ml) alone for an additional 14 days. This interval compels the glands to undergo a structural regression back to the morphological appearance of glands resembling virgin mice. The glands for MDL are fixed in formalin for 24 h and processed for histopathological evaluation. The glands for MAL are fixed and stained with alum carmine for evaluation of unregressed areas and evaluated for the incidence (number of glands with lesions/total number of glands). For MDL, the glands are divided into several microscopic fields and each field is analysed for ductal sections; the ducts containing lesions are compared with the total number of ductal fields counted to determine the incidence of MDL.

In order to determine whether the effects of 1α (OH)D5 were selective for the initiation or promotion phases of lesion formation,10 glands per group were incubated with the vitamin D analogue for the first four days (0–4 days) of culture, which includes 3 days prior to DMBA treatment of the glands and one day post-DMBA treatment. For determining the antipromotional effects of 1α (OH)D5, the glands were incubated with 1 μM 1α (OH)D5 from day 4 to day 10 of the growth-promoting phase of epithelial cells.

2.2. Mammary carcinogenesis experiment

Fifty-day-old Sprague Dawley female rats were used for the study. All procedures were carried out under institutional guidelines and an approved protocol. Animals were randomised by weight into four groups of 20 animals each and received 15 mg of DMBA in 1 ml of corn oil intragastrically. The groups' diets included: (1) a placebo diet (the control group); (2) a diet supplemented with 1α (OH)D5 from 2 weeks prior to carcinogen treatment until the end of the study (initiation + promotion phases, −2 to end of study); (3) a 1α (OH)D5 supplemented diet from 2 weeks prior to the carcinogen treatment to the week after the carcinogen treatment (initiationphase only −2 to +1 week); and (4) a 1α (OH)D5-diet beginning one week after the carcinogen treatment until the end of the study (promotion phase only, +1 to end of study). Two additional groups were also included with 10 rats per group receiving no carcinogen and either the placebo or the 1α (OH)D5 supplemented diet. The concentration of 1α (OH)D5 in the diet was kept at 40 μg/kg diet. Beginning 3 weeks after the carcinogen treatment and continuing until the end of the study, the animals were weighed once a week and examined weekly by palpation. All animals were sacrificed 150 days post-carcinogen treatment. Tumours were removed and processed for histopathology and a portion of the tumour was saved for biochemical analyses.

2.3. Statistical analysis

Statistical significance between groups in MMOC was determined by χ^2 analysis. Tumour incidence in the carcinogenesis experiment was evaluated by unpaired Students *t*-test and χ^2 analysis. Latency for tumour appearance was determined by an analysis of variance test(ANOVA).

3. Results

As shown in Fig. 1, the basic difference between the natural active metabolite of vitamin D, 1,25 dihydroxyvitamin D3(1,25(OH)2D3), and 1α-hydroxyvitamin D5(1α(OH)D5) is that there is no hydroxylation at the 25 position in the D5 analogue, instead there is an ethyl group in the C-24 position of the vitamin D3 molecule. Both of these molecules are different in their retention properties on a high-performance liquid chromatography(HPLC) column. 1,25(OH)2D3 separated with a retention time of 5.2 min compared with 34.0 min for 1α (OH)D5. In the presence of insulin, prolactin, aldosterone and hydrocortisone MAL were induced in response to DMBA. In three experiments with15 glands per experiment, MAL were observed in 30 glands out of 45 (67% incidence). Incubation of glands in the presence of 1 μM 1α (OH)D5 resulted in a >60% suppression of MAL incidence. Out of 45 glands, 11 exhibited MAL, a 63% suppression of MAL development ($P < 0.001$). A representative photograph showing MAL morphology in a DMBA-treated gland compared with a control gland not treated with DMBA and a chemopreventive agent-treated gland is shown in Fig. 2. The ductal lesions were induced by including 0.001 μg/ml oestradiol and 1 μg/ml progesterone in the medium. These steroid hormones replaced aldosterone and hydrocortisone in the medium. 22 of 32 ductal sections examined in the

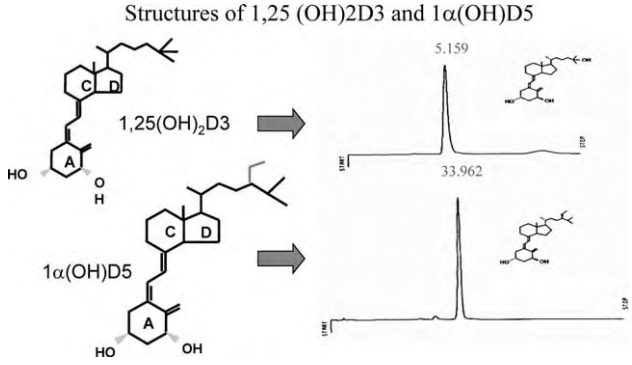


Fig. 1. Chemical structures of vitamin D analogues. Structural differences between 1,25 dihydroxyvitamin D3 (1,25(OH)2D3) and 1α-hydroxyvitamin D5(1α(OH)D5) are shown. High-performance liquid chromatographic (HPLC) analyses to show different retention times are shown as HPLC profiles for these two agents.

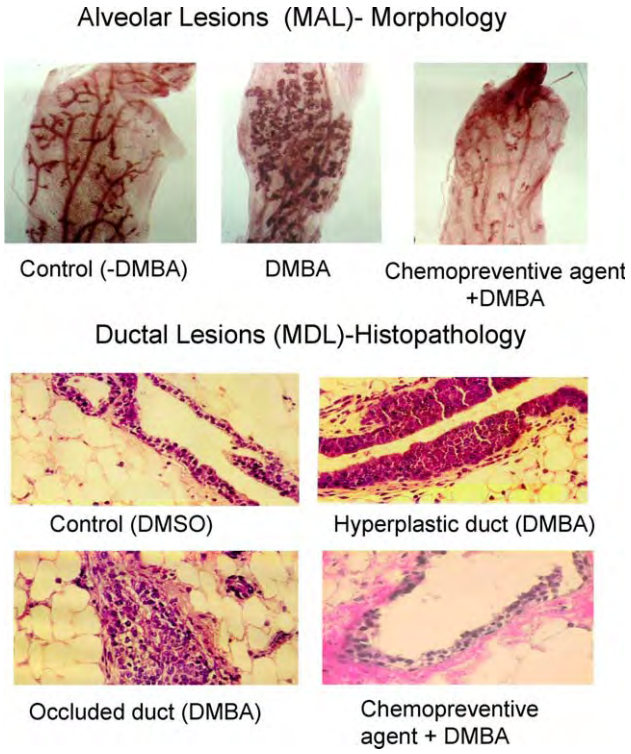


Fig. 2. Representitative examples of mammary alveolar and mammary ductal lesions in response to 7,12-dimethylbenz(*a*)anthracene (DMBA) and efficacy of chemopreventive agent. Thoracic pairs of mammary glands were dissected from oestrogen-and progesterone-pretreated mice. The glands were incubated with either aldosterone and hydrocortisone (for ovarian hormone-independent mammary alveolar lesions (MAL)) or with oestrogen and progesterone (for ductal lesions) for 10 days. The carcinogen treatment was for 24 h on day 3. The growth-promoting hormones were removed from the medium, leaving only insulin for additional 14 days. The chemopreventive agent was present for the first 10 days along with the growth promoting hormones. Glands were either stained with alum carmine (for MAL) or sectioned for histopathological processing and stained (for mammary ductal lesions (MDL)). The ovarian hormone-independent alveolar lesions are shown in the upper panels. The representative photographs of ovarian hormone-dependent ductal lesions and response to chemopreventive agent are shown in histopathological sections.

control glands contained hyperproliferative and atypical ductal lesions (Table 1). Treatment of the glands with 1 μM 1α (OH)D5 resulted in the suppression of these ductal lesions and only 6 of 24 ductal sections showed the presence of MDL. Representative photographs showing MDL and effects of a chemopreventive agent are shown in Fig. 2. These results indicated that there was a 64% inhibition of MDL formation by 1 μM D5 treatment ($P < 0.001$). These results suggest that 1α (OH)D5 inhibited the development of both ovarian hormone-independent (MAL) and hormone-dependent (MDL) mammary lesions. (Table 1)

In order to evaluate the stage-specific efficacy of 1α (OH)D5 on the development of DMBA-induced MAL formation, 15–20 glands per group were incubated with a MAL-promoting hormone combination, with or without 1 μM 1α (OH)D5. The D5-analogue was included in the medium during either the initiation phase from 0 to 4 days of culture (DMBA on day 3) or the promotion phase from day 4 to day 10 of the culture period. The control glands in this series of experiments developed MAL in 60% (18 out of 30 glands) of the glands. Compared with controls, treatment of glands during the initiation phase resulted in 12 out of 30 (40%) glands developing lesions. An inhibition rate of 33% [1 − (40% treated glands/60% controls) × 100]. This anti-initiation effect of 1α (OH)D5 was not statistically significant ($P > 0.1$). On the other hand, anti-promotional effects resulted in 6 glands with MAL out of 30 in the culture (20%). Comparison of treated and control lesion incidence indicated that there was a 67% ($P < 0.001$) inhibition in the promotion stage of MAL formation (Table 1).

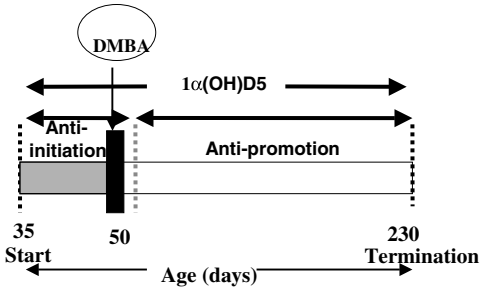
Previously, we showed that 1α (OH)D5 (50 μg/kg diet) inhibited both the incidence and multiplicity of MNU-induced mammary carcinogenesis in rats [23]. In this study, we evaluated the efficacy of 1α (OH)D5 at a 40 μg/kg diet concentration on DMBA-induced mammary carcinogenesis during the initiation and promotion phases. As shown in Table 2, DMBA induced mammary tumours in 16/20 animals, resulting in a tumour incidence of 80%. When the chemopreventive agent was present in the diet beginning 2 weeks before the carcinogen treatment and continued throughout the experimental period (initiation plus promotion), there was a reduction in tumour incidence from 80 to 50 percent (a 37.5% inhibition of incidence). This reduction in tumour incidence was compared with the initiation and promotion phases individually. In the group where the vitamin D analogue was included in the diet for a short period of 3 weeks, from 2 weeks prior to DMBA treatment to one week after, tumour incidence was 70%, a 12.5% reduction. These results suggested that 1α (OH)D5 had very little effect on the initiation of carcinogenesis in this model. Results also showed that when the chemopreventive agent was present in the diet from one week after the carcinogen treatment until the end of the study, tumour incidence was again 50%, a reduction of 37.5% ($p < 0.05$). This reduction was the same as when the treatment period included both the initiation and promotion phases. These results suggest that the effect of vitamin D may be selective during the promotion phase of carcinogenesis. Moreover, the median time for the appearance of the first tumour (latency) was compared amongst all groups and statistically analysed using ANOVA. The time to the first tumour appearance after the carcinogen treatment was 92.5 ± 5.9 days for the control group as compared with 120.7 ± 9.4 days for the promotion group (+1 to end). These results once again suggest a trend towards increased latency when 1α (OH)D5 was included during

Table 1
Chemopreventive efficacy of 1α-Hydroxyvitamin D5 in organ cultures

Treatment (DMBA + D5)	Days	Number of glands with lesions (% incidence)	% Inhibition	Ductal sections with lesions (% multiplicity)	Percent inhibition	Significance (P values[a])
Mammary alveolar lesions (MAL)						
None	N/A	30/45 (67)	–	N/A	N/A	
10–7M D5	0–10	14/30 (47)	30	N/A	N/A	0.085
10–6M D5	0–10	11/45 (24)	63	N/A	N/A	0.001
None	N/A	18/30 (60)		N/A	N/A	
10–6M D5	0–4	12/30 (40)	33	N/A	N/A	0.12
10–6M D5	4–10	6/30 (20)	67	N/A	N/A	0.0016
Mammary ductal lesions (MDL)						
None	N/A	N/A	N/A	22/32 (69)	–	
10–6M D5	0–10	N/A	N/A	6/24 (25)	64	0.0012

N/A, Not applicable.
[a] χ^2 analysis, comparision with controls.

Table 2
Effects of 1(OH)D5 in DMBA-induced mammary carcinogenesis in rats



Treatment	N	Schedule (wks)	Incidence (%)	Latency (days)	Final BW (g)
Control	20	–	16/20 (80)	92 ± 5	265 ± 10
D5 (40 μg/kg)	20	−2 to End	10/20 (50)*	106 ± 11	254 ± 13
D5 (40 μg/kg)	20	−2 to +1	14/20 (70)	114 ± 8	266 ± 8
D5 (40 μg/kg)	20	+1 to End	10/20 (50)*	121 ± 9	255 ± 11

wks, weeks; BW, body weight.
* $p < 0.05$ (compared with controls).

the post-carcinogen treatment phase. However, latency for the appearance of the first tumour amongst groups was not statistically significant. There was no difference observed in body weight gains. The initial weight was similar in all animals since they were randomised in groups based on their average weights. The final body weights for all groups are shown in Table 2. There was no difference in serum calcium or phosphorous concentrations, indicating no hypercalcaemic activity (data not shown).

4. Discussion

Although vitamin D has been considered as one of the most effective differentiating agents in leukaemic cells and as an antiproliferative agent against many cancer cells including breast, prostate and colon cancers [5,6], its clinical use has been limited due to the known hypercalcaemic activity of the natural metabolite of vitamin D3, 1,25(OH)2D3. Recognising the possible translational value of vitamin D, more than 1000 analogues of vitamin D3 have been synthesised in the past 15 years and many of them have been evaluated for their possible antiproliferative activity at non-toxic concentrations. We synthesised 1α (OH)D5 as an analogue of the D5 class of vitamin, which is also a modification of cholecalciferol [22], and evaluated its efficacy as an antiproliferative agent against breast cancer cells in culture. Results showed that it was efficacious against both ER+ and ER− breast cancer cells so long as they are VDR positive (VDR+) [26]. Simultaneously, we also

showed that 1α (OH)D5 could be tolerated by rats and mice at higher concentrations than most of the other vitamin D analogues. Toxicity studies with 1α (OH)D5 in dogs and rats have been completed under good laboratory practice (GLP) guidelines and it will be evaluated in a Phase I study for breast cancer patients. It should also be noted that a high concentration of 1α (OH)D5 is required to produce protective effects. However, the analogue is non-calcaemic at an effective concentration. Compared with EB1089, RO24–5531 and 1,25(OH)2D3, 1α (OH)D5 requires a log molar higher concentration to provide equivalent effects in cell cultures. Most effective analogues are active at 10–7M concentrations, whereas D5 requires a 1 μM concentration to have antiproliferative effects. At 1 μM concentration, 1α (OH)D5 inhibited the development of MAL in MMOC.

More recently, we showed that if glucocorticoids in the medium are replaced with oestradiol 17β and progesterone, glands develop ductal lesions. These ductal lesions can be suppressed by tamoxifen; however, tamoxifen was ineffective against MAL, indicating these lesions have different properties in relation to hormonal sensitivity [27,25]. In the present study, we observed that 1α (OH)D5 is efficacious against both alveolar (ovarian hormone-independent) and ductal (oestradiol 17β-progesterone dependent) lesions. This is consistent with a prior report that indicated that the effect of 1α (OH)D5 was dependent on VDR in the cells. The protective role of VDR in the mammary gland has been recently evaluated by examining mammary gland development in VDR-knockout (KO) mice. Results showed that the glands from VDR–KO mice exhibited accelerated growth, and the regression of the gland during involution was observed at a reduced rate [28]. In MMOC, it was observed that VDR sensitised mammary glands to 1,25(OH)2D3, because the glands from VDR–KO mice did not respond to the vitamin D analogue [29]. Both ER+ and ER− breast cancer cells that were VDR+ responded to vitamin D analogues [26]. MMOC can be expanded to determine if the chemopreventive agent is selectively effective against either the initiation or promotion stage by exposing the glands to the test agent either before or after the carcinogen treatment. In the present study, we found that 1α (OH)D5 showed efficacy selectively against the promotion stage of lesion formation: there was more suppression of alveolar lesions during the promotion stage than during the initiation stage.

Numerous chemopreventive agents have been evaluated in MMOC and rat mammary carcinogenesis protocols [30]. Results have shown that there is a 75% correlation between efficacy observed in MMOC and efficacy *in vivo*. Several vitamin D analogues, including EB1089 [31], RO24–5531 [32] and 1α, 25(OH)2D3 [33], have been reported to have chemopreventive activity in mammary carcinogenesis. The question has been asked as to whether 1α (OH)D5 can be detected in mammary tissues subsequent to *in vivo* treatment. We determined the tissues level of 1α (OH)D5 in rats. Rats were treated with 50 μg (1.2 μmoles) of 1α (OH)D5 at 50 days of age for 24 h. Mammary glands were removed and extracted for vitamin D. The extract was separated on liquid chromatography-mass spectrometry (LC-MS) using 1α (OH)D5 as a standard. Results showed that there was 3.15ng (63 pmoles)/mg mammary gland. It is not possible to make a direct comparison between the amount of 1α (OH)D5 included in the medium (1 μM or 428ng/ml) and its *in vivo* uptake by the tissues. Nonetheless, 1α (OH)D5 by itself was detected in the mammary tissue.

Earlier, we reported that in an MNU-induced mammary carcinogenesis model, 1α (OH)D5 inhibited the incidence of mammary tumour development in adult (100 day old) rats. In the present experiment, we evaluated the effectiveness of 1α (OH)D5 in a DMBA-induced carcinogenesis model using 50-day-old rats. The results are comparable to those observed for MNU-induced cancers. At a 40 μg/kg D5 diet level, there was a reduction in the incidence of tumour development. There was a 37.5% ($p < 0.05$) reduction in tumour incidence in groups receiving D5 either during the promotion phase (+1 to end) alone or during the entire period of carcinogenesis (−2 to end). However, if the animals consumed D5 for a short time only (−2 to +1 weeks), the reduction in tumour incidence was marginal (12.5%). These *in vivo* results are consistent with the MMOC results described in this report. It would be of considerable importance if a chemopreventive agent suppresses the latency of the tumour occurrence. However, in the present study, there was no statistically significant increase in the median latency times observed in any of the groups. These results clearly support developing 1α (OH)D5 for further studies and clinical trials.

Conflict of Interest

None.

Acknowledgements

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Inhibition of Proliferation and Induction of Apoptosis by 25-Hydroxyvitamin D_3-3β-(2)-Bromoacetate, a Nontoxic and Vitamin D Receptor-Alkylating Analog of 25-Hydroxyvitamin D_3 in Prostate Cancer Cells

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ABSTRACT

The 25-hydroxyvitamin D_3 (25-OH-D_3) is a nontoxic and low-affinity vitamin D receptor (VDR)-binding metabolic precursor of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. We hypothesized that covalent attachment of a 25-OH-D_3 analog to the hormone-binding pocket of VDR might convert the latter into transcriptionally active holo-form, making 25-OH-D_3 biologically active. Furthermore, it might be possible to translate the nontoxic nature of 25-OH-D_3 into its analog. We showed earlier that 25-hydroxyvitamin D_3-3-bromoacetate (25-OH-D_3-3-BE) alkylated the hormone-binding pocket of VDR. In this communication we describe that 10^{-6} mol/L of 25-OH-D_3-3-BE inhibited the growth of keratinocytes, LNCaP, and LAPC-4 androgen-sensitive and PC-3 and DU145 androgen-refractory prostate cancer cells, and PZ-HPV-7 immortalized normal prostate cells with similar or stronger efficacy as 1,25$(OH)_2D_3$. But its effect was strongest in LNCaP, PC-3, LAPC-4, and DU145 cells. Furthermore, 25-OH-D_3-3-BE was toxic to these prostate cancer cells and caused these cells to undergo apoptosis as shown by DNA-fragmentation and caspase-activation assays. In a reporter assay with COS-7 cells, transfected with a 1α,25-dihydroxyvitamin D_3-24-hydroxylase (24-OHase)-construct and VDR-expression vector, 25-OH-D_3-3-BE induced 24-OHase promoter activity. In a "pull down assay" with PC-3 cells, 25-OH-D_3-3-BE induced strong interaction between VDR and general transcription factors, retinoid X receptor, and GRIP-1. Collectively, these results strongly suggested that the cellular effects of 25-OH-D_3-3-BE were manifested via 1,25$(OH)_2D_3$/VDR signaling pathway. A toxicity study in CD-1 mice showed that 166 μg/kg of 25-OH-D_3-3-BE did not raise serum-calcium beyond vehicle control. Collectively, these results strongly suggested that 25-OH-D_3-3-BE has a strong potential as a therapeutic agent for androgen-sensitive and androgen-refractory prostate cancer.

INTRODUCTION

Alkylating agents, such as estramustine, lomustine, procarazine, busulfan, cyclophosphamide, and chlorambucil, platinum coordination complexes are important components in the standard cancer chemotherapeutic regimen. However, majority of these drugs are nonspecific and produce significant to severe side effects, particularly at doses required for the reduction/elimination of tumor (1). Affinity alkylating compounds, on the other hand, cross-link to the substrate/ligand-binding sites of target enzymes/receptors; thus, they can potentially modulate the biological property associated only with the target molecule/molecules (2). We postulated that such target specificity might lower the therapeutic dose of the compounds and can potentially avoid harmful side effects.

Vitamin D receptor (VDR), the nuclear receptor for the vitamin D hormone, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is a known target for the potential development of anticancer drugs (3–5). The main obstacle in such efforts has been toxicity of 1,25$(OH)_2D_3$, and many of its synthetic analogs related to hypercalcemia, particularly at doses to have a beneficial effect. The cell-regulatory properties of 1,25$(OH)_2D_3$ and its synthetic analogs are associated with the activation of VDR, but a similar link with calcemic activity is yet to be established firmly. A robust effort has been underway to develop vitamin D derivatives with strong antiproliferative property and reduced toxicity. This effort has produced many vitamin D analogs; and it has been possible to dissociate, at least in part, hypercalcemia from antiproliferative properties in certain analogs, classified as "noncalcemic vitamin D analogs" (6). EB-1089, one such analog, is currently in clinical trials for breast, colorectal, pancreatic, and hepatocellular carcinomas (7–11). Such success has provided a strong impetus to addi-

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tionally develop therapeutically important vitamin D analogs for a broad range of diseases, including cancer.

The 25-hydroxyvitamin D_3 (25-OH-D_3), the metabolic precursor of 1,25$(OH)_2D_3$, has a significantly reduced VDR-binding affinity. As a result, 25-OH-D_3 is not considered to be biologically active. Additionally, it is nontoxic [serum concentration of 25-OH-D_3 is 40 to 100 ng/mL *versus* 8 to 10 pg/mL for 1,25$(OH)_2D_3$]. We hypothesized that if 25-OH-D_3 could be covalently attached to the hormone-binding pocket of *apo*-VDR, it might be possible to convert the latter into transcriptionally active *holo*-form. This would make 25-OH-D_3 biologically active. Furthermore, it might be possible to translate the nontoxic nature of 25-OH-D_3 into its VDR-alkylating analog. Recently, we showed that 25-hydroxyvitamin D_3-3β-(2)-bromoacetate (25-OH-D_3-3-BE), a derivative of 25-OH-D_3, specifically alkylated the hormone-binding pocket of VDR (12). Therefore, 25-OH-D_3-3-BE became an ideal candidate to validate our hypothesis.

In the present study, we investigated the effect of 25-OH-D_3-3-BE in a set of normal and malignant cell lines and observed that antiproliferative property of 25-OH-D_3-3-BE was most pronounced in prostate cancer cells. In addition, we observed that 25-OH-D_3-3-BE caused apoptosis in prostate cancer cells; an observation supported by DNA fragmentation and caspase-activation studies. Mechanistic studies showed that the effects of 25-OH-D_3-3-BE were mediated by VDR. Moreover in a CD-1 mouse model, it was observed that 25-OH-D_3-3-BE did not raise serum calcium beyond control at doses considered to be highly toxic for 1,25$(OH)_2D_3$ and many of its synthetic analogs. Results of these studies and their implications are discussed in this communication.

MATERIALS AND METHODS

The 25-OH-D_3-3-BE was synthesized according to our published procedure (13). The majority of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless mentioned otherwise. The hVDR expression vector pAVhVDR was a generous gift from Dr. Wesley Pike (University of Wisconsin, Madison, WI). All of the cell lines were obtained from American Type Culture Collection (Manassas, VA), except LAPC4 cells that were obtained from the laboratory of Charles Sawyers (Department of Medicine, University of California at Los Angeles, Los Angeles, CA).

Male CD-1 mice 6 to 8 weeks old, average weight 30 g were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in cages of five (5) in a group and were fed rat chow and water ad lib. Animal experiment was carried out in the animal facility of Boston University School of Medicine with strict adherence to the guidelines of Laboratory Animal Safety Committee. Serum calcium values in blood samples were determined at the Core Chemistry Laboratory of Boston University Medical Center.

Cell Culture. PZ-HPV-7 cells were grown in MCDB media containing pituitary extract, epidermal growth factor, and 1% penicillin/streptomycin. Keratinocytes were also grown in the same media with additional PG1 and insulin. PC-3, LNCaP, and DU-145 cells were grown in RPMI containing 10% fetal bovine serum (FBS) and antibiotics. MCF-7 cells were grown in DMEM containing 10% FBS and antibiotics. LAPC-4 cells were maintained in IMEM containing antibiotics including 1% L-glutamine and 10 nmol/L of R1881, a synthetic progestin. MC3T3 cells were grown in αMEM containing 10% FBS and antibiotics. In general, cells were grown in 35-mm dishes to 70 to 80% confluence and then plated into 24-well plates in respective media. After the cells grew to ~70% confluence, they were serum-starved for 20 hours (PC-3, LNCaP, and DU-145 cells) followed by incubation with steroid samples. Keratinocytes and PZ-HPV-7 cells, after reaching 70% confluence, were kept in MCDB media without additives for 20 hours before treatment with steroids. In general, reagents were dissolved in EtOH, and dilution with the media was adjusted in such a way that concentration of EtOH was 0.1% v/v.

In a separate experiment (cell counting), LAPC-4, LNCaP, MCF-7, and MC3T3 cells were grown to desired confluence and treated with the reagents (without serum starvation) for 24 hours (LNCaP, MC3T3, and LAPC-4) or 48 hours (MCF-7) with EtOH vehicle or 25-OH-D_3-3-BE (10^{-6} mol/L) or 1,25$(OH)_2D_3$ (10^{-7} mol/L). At the end of the experiment, cells were detached with trypsin-EDTA and counted in a Coulter counter.

Keratinocytes, procured from neonatal foreskin after overnight trypsinization at 4°C and treatment with 0.2% EDTA, were grown in culture with a modification of the published method (14). The 3T3 cells were plated at 10^4 cells/35-mm tissue-culture dish and were irradiated lethally after 2 days with a ^{60}Co source (5,000 rads). Keratinocytes, in 1 mL serum-free medium, were plated on lethally irradiated 3T3 cells. When these cells reached ~70% confluence, they were plated onto 24-well plates. Each experiment was done on primary or secondary keratinocyte cultures obtained from different skin samples.

The [^{3}H]Thymidine Incorporation Assay. In a typical assay, cells were grown to 60 to 70% confluence in 24-well plates in respective media containing 10% FBS, and serum starved for 20 hours, followed by treatment with various agents (in 0.1% ethanolic solution) or EtOH (vehicle) in serum-containing medium for 16 to 18 hours. After the treatment, media was removed from the wells and replaced with media containing [^{3}H]thymidine (0.1μCi) per well, and the cells were incubated for 3 hours at 37°C. After this period, media was removed by aspiration, and the cells were washed thoroughly (3 × 0.5 mL) with PBS. Then ice-cold 5% perchloric acid solution (0.5 mL) was added to each well, and the cells were incubated on ice for 20 minutes. After this incubation, perchloric acid was removed by aspiration, replaced with 0.5 mL of fresh perchloric acid solution, and the cells were incubated at 70°C for 20 minutes. Solution from each well was mixed with scintillation fluid and counted in a scintillation counter.

Majority of these assays were carried out in six (6) replicates with 10^{-6} mol/L of reagents. In the dose-response study, PC-3 cells were incubated with EtOH or 10^{-7} to 10^{-6} mol/L of 25-OH-D_3-3-BE or 1,25$(OH)_2D_3$ for 18 hours followed by [^{3}H]thymidine incorporation assay described above.

The 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) Cell Viability Assay. LNCaP, PC-3, and DU145 cells were plated in 96-well plates (7,500 cells per well), grown overnight in DMEM (with 10% FBS and antibiotics), and serum deprived for 24

hours. The cells were then treated with either EtOH or $1,25(OH)_2D_3$ (10^{-6} mol/L) or 25-OH-D_3-3-BE (10^{-6} mol/L) for 18 hours in complete media. Cell viability was measured with the CellTiter 96 AQueous Assay (Promega, Madison, WI). This assay used the tetrazolium compound (MTS, inner salt) and the electron-coupling reagent, phenazine methosulfate (15). This assay measured dehydrogenase enzyme activity found in metabolically active cells, which reduced MTS into soluble and colored formazan product, absorbance of which was measured at 490 nm. Because the production of formazan was proportional to the number of living cells, absorbance was a measure of cell-viability.

DNA-Fragmentation Analysis. PC-3 cells (2×10^6) were treated with 0.25×10^{-6} mol/L of $1,25(OH)_2D_3$, 25-OH-D_3, or 25-OH-D_3-3-BE for 10 hours. Then the cells were harvested and lysed in 0.5 mL of lysis buffer [20 mmol/L Tris-HCl, 10 mmol/L EDTA, 0.5% Triton X-100 (pH 8.0)], and DNA was extracted with phenol-chloroform procedure. The extracted DNA was resuspended in 0.1 mL of 20 mmol/L Tris-HCl (pH 8), and treated with RNase, followed by electrophoresis on a 1.2% agarose gel in TAE buffer. DNA bands were visualized under UV light after ethidinium bromide staining.

Caspase Activity. Caspase-3, -8, and -9 assays were done with Caspase colorimetric assay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. Briefly, PC3 cells (1×10^6) were treated with 0.01×10^{-6} mol/L of $1,25(OH)_2D_3$, 25-OH-D_3, or 25-OH-D_3-3-BE for 14 hours in culture medium (DMEM, 10% FBS, and antibiotics). The cells were collected by centrifugation at 1,000 rpm for 5 minutes. The cell pellet was lysed with lysis buffer, and the lysate was incubated on ice for 10 minutes and centrifuged at 10,000 rpm for 5 minutes. Protein was estimated with Bradford protein estimation kit (Bio-Rad Laboratories, Hercules, CA). The enzymatic reactions were carried out in a 96-well plate. For each reaction, 100 μg lysate protein in 50 μL total volume was incubated with 50 μL of 2 × reaction buffer and 5 μL of caspase 3, caspase 8, or caspase 9 colorimetric substrates for 2 hours at 37°C. The absorbance was determined at 405 nm.

Induction of 1α,25-Dihydroxyvitamin D_3-24-Hydroxylase (24-OHase) Promoter Activity by 25-OH-D_3-3-BE and $1,25(OH)_2D_3$ in COS-7 Cells

Cell Transfections. Promoter constructs containing the rat 24-OHase promoter (−1,367/+74) linked to the chloramphenicol acetyltransferase (CAT) reporter gene were used for the experiment. COS-7 cells that were transfected with the hVDR expression vector pAVhVDR. All of the transfections were done with the calcium phosphate DNA precipitation method. The COS-7 cells were seeded with 1×10^6 cells/100 mm^2 tissue culture plate in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and allowed to grow for 18 to 20 hours or to 70 to 80% confluency. The DNA to be transfected was EtOH-precipitated. For each plate to be transfected, 450 μL of sterile ddH_2O and 50 μL of 2.5 mol/L $CaCl_2$ were added to the DNA pellet. This mixture was then added to 500 μL of 2 × HEPES buffer per sample dropwise while mixing. After the two solutions were combined, the resulting mixture was vortexed and allowed to sit at room temperature for 20 minutes to allow the DNA to precipitate. Finally, the DNA precipitate was mixed thoroughly, and 1 mL aliquots were added to each plate. Sixteen hours post transfection, cells were "shocked" for 1 minutes with PBS containing 10% dimethylsulfoxide, washed with PBS, and the DMEM supplemented with 2% of charcoal dextran-treated FBS was added to each plate. The cells were then treated with various doses of $1,25(OH)_2D_3$ or 25 OH-D_3-3-BE for 24 hours.

CAT Assay. Treated cells were harvested by trypsinization for about 2 minutes at 37°C, pelleted, washed with PBS, resuspended in 0.25 mol/L Tris-HCl (pH 8.0), and lysed by freezing and thawing five (5) times. Cellular extracts were collected and used for CAT assays.

CAT analysis was done by standard protocols on the cell extracts normalized to total protein content. Fifty microliters aliquots of cellular extracts containing equal amounts of protein were combined with 25 μL of 1 mol/L Tris-HCl (pH 8.0), 53 μL of ddH_2O, 20 μL of 4 mmol/L acetyl CoA, 2 μL of ^{14}C chloramphenicol (50 mCi/mmol; Sigma, St. Louis, MO), and 0.25 mmol/L Tris-HCl (pH 8.0) to a final volume of 150 μL. The reactions were carried out at 37°C for about 2 hours and stopped by adding 1 mL of ethyl acetate and vortexing. The samples were centrifuged at 14,000 rpm at 4°C for 10 minutes, and the upper ethyl acetate layer was removed to a microcentrifuge tube and dried under vacuum for 45 minutes. The samples were resuspended in 25 μL of ethyl acetate and spotted on a TLC plate. Chromatography was done in a chromatography chamber containing 100 mL of chloroform-methanol (95:5) for 40 minutes. The plate was dried and exposed to Kodak autoradiographic film. The resulting autoradiogram was analyzed by densitometric scanning with the Shimadzu CS9000U Dual-wavelength Flying Spot Scanner (Shimadzu Scientific Instruments, Princeton, NJ).

Pull Down Assays to Determine the Interaction of VDR with Retinoid X Receptor (RXR) and GRIP-1 in the Presence of $1,25(OH)_2D_3$ or 25-OH-D_3-3-BE in PC-3 Cells. In this assay, PC-3 cells were incubated for either 1 or 24 hours with the indicated concentrations of $1,25(OH)_2D_3$ or 25-OH-D_3-3-BE, and then the cells were scraped, homogenized, and whole-cell extracts were prepared in NETND buffer [100 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 7.8), 0.2% NP40, and 1 mmol/L dithiothreitol] containing 0.3 mol/L KCl. Then, 5 μg of purified glutathione *S*-transferase (GST) fusion protein (GST-GRIP or GST-RXR), and 20 μL of glutathione-Sepharose beads were added, and the volume was brought up to 100 μL with the same buffer. These mixtures were incubated for 1 or 24 hours at 4°C, and the beads were washed 3 times with 0.2 mL of NETND buffer. The bound proteins were eluted from the packed beads by boiling in Laemmli buffer for 3 minutes and were analyzed by SDS-PAGE. Detection of "bound-VDR" was done after SDS-PAGE by Western blots with VDR antibodies (Affinity BioReagents, Golden CO).

Determination of Systemic Toxicity (Calcemia) of 25-OH-D_3-3-BE in CD-1 Mice. Three doses of 25-OH-D_3-3-BE (3.3, 33, or 166.7 μg/kg) and two doses (3.3 or 33 μg/kg) of 25-OH-D_3 were prepared in 0.2 mL of saline-EtOH (0.1%) by diluting ethanolic solutions of the steroids with saline in such a way that the concentration of EtOH was 0.1% in the solution. These samples or saline-EtOH (0.1%) vehicle control (0.2 mL) were administered to the animals (in groups of five) intraperi-

toneally over a period of 12 days (injection on alternate days). At the end of the experiment the animals were lightly anesthetized, and blood was collected after decapitation for serum calcium-analysis. Body weights at the beginning and at the end of the experiment were recorded.

Data Analysis. Majority of the assays was carried out in three to six replicates. Statistical analyses of the data were done with linear regression analysis and one-way ANOVA followed by Fisher's protected least significant difference tests. *P*s ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The $1,25(OH)_2D_3$ and many of its synthetic analogs inhibit the growth of malignant cells (16). However, translation of the cellular results into *in vivo* studies has been problematic because of acute toxicity of the hormone and some of its analogs. Therefore, a major effort has been underway in designing analogs that would either inhibit cellular growth at physiologic concentrations to avoid systemic toxicity or be nontoxic at supraphysiologic doses.

VDR-binding affinity is crucial in developing $1,25(OH)_2D_3$ analogs because of the recognition that interaction between VDR and the analogs is pivotal in the genomic process (3, 4). Therefore, analogs with relatively low VDR-binding affinity have not been considered to be of therapeutic importance. For example, 25-OH-D_3, the metabolic precursor of $1,25(OH)_2D_3$, has a poor VDR-binding affinity [$K_d = 10^{-6}$ to 10^{-7} mol/L *versus* $K_d = 10^{-9}$ to 10^{-10} mol/L for $1,25(OH)_2D_3$]. Therefore, 25-OH-D_3 and its derivatives have not been studied significantly as candidates for drug-development.

We hypothesized that covalent linking of 25-OH-D_3 (via its derivative/analog) to the hormone-binding pocket of VDR might permanently lock VDR into its biologically active *holo*-form. This way, biologically inactive 25-OH-D_3 might acquire significant cell regulatory property. Furthermore, because of the recognition that calcemic property could be separated from cell regulatory properties, the nontoxic property of 25-OH-D_3 might be translated into its derivative. As a result, even supraphysiologic doses of this 25-OH-D_3 analog might be used to achieve inhibition of cell growth without systemic toxicity in an *in vivo* system.

In a recent, study we showed that 1α,25-dihydroxyvitamin D_3-3β-(2)-bromoacetate [$1,25(OH)_2D_3$-3-BE], an affinity labeling derivative of $1,25(OH)_2D_3$, displayed strong antiproliferative effect in several normal and malignant cell lines with strongest activity toward prostate cancer cells (17–21). In the current study, we focused on a structural 25-OH-D_3-prototype of $1,25(OH)_2D_3$-3-BE (*i.e.*, 25-OH-D_3-3-BE).

Growth-inhibitory effect of $1,25(OH)_2D_3$ and its analogs is known to vary among cell lines and even among lines from the same tissue. But, in general, strongest and predictable effect is observed at a 10^{-6} mol/L concentration of the hormone or its analogs (22). Although this concentration is considered to be physiologically irrelevant, it produces optimal effect. We treated primary culture of normal human skin cells, and several cell lines including LNCaP human androgen-sensitive and PC-3 human androgen-refractory prostate cancer cells, and PZ-HPV-7 immortalized normal human prostate cells with 10^{-6} mol/L of $1,25(OH)_2D_3$ or 25-OH-D_3-3-BE to compare the antiproliferative property of the analog (25-OH-D_3-3-BE) with the hormone.

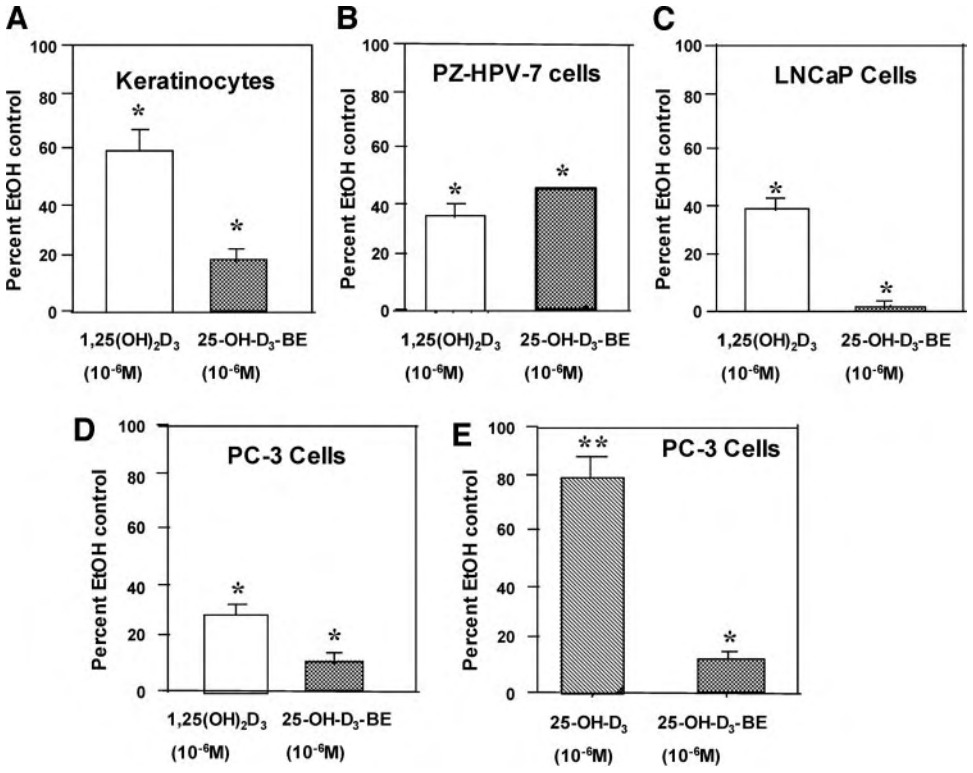

Fig. 1 [^{3}H]Thymidine incorporation assays of keratinocytes, PC-3, LNCaP, and PZ-HPV-7 cells. Cells, grown to 60 to 70% confluence were serum starved for 20 hours followed by treatment with 10^{-6} mol/L of 25-OH-D_3-3-BE, $1,25(OH)_2D_3$, 25-OH-D_3, or EtOH (control) for 16 hours followed by incubation with [^{3}H]thymidine and assaying for the incorporation of radioactivity in the cells. Results are expressed relative to EtOH control (100%). *, $P < 0.00032$; **, $P < 0.0075$. Bars, ±SD.

Effect of various agents on the growth of normal and malignant cells is often determined by [^{3}H]thymidine incorporation assay. In this assay, increase or decrease in the incorporation of [^{3}H]thymidine in the DNA of the growing cells by a reagent is used as an index of its proliferative/antiproliferative effect. As shown in Fig. 1, *A–E*, 10^{-6} mol/L of 25-OH-D_3-3-BE and 1,25$(OH)_2D_3$ inhibited the growth of all the cells with various efficiency. However, the effect of 25-OH-D_3-3-BE was strongest in LNCaP and PC-3 prostate cancer cells. For example, growth of LNCaP cells were inhibited by ~60% and 98% with 1,25$(OH)_2D_3$ and 25-OH-D_3-3-BE, respectively (Fig. 1*C*), whereas growth of PC-3 cells were retarded by 70% and 90% by 1,25$(OH)_2D_3$ and 25-OH-D_3-3-BE, respectively (Fig. 1*D*). In contrast, growth of normal immortalized prostate cells (PZ-HPV-7 cells) were inhibited by ~50% and 65% by 10^{-6} mol/L of 25-OH-D_3-3-BE and 10^{-6} mol/L of 1,25$(OH)_2D_3$, respectively (Fig. 1*B*). Growth inhibition by 25-OH-D_3-3-BE was stronger than an equivalent amount of 1,25$(OH)_2D_3$ in keratinocytes (Fig. 1*A*). Furthermore, 10^{-6} mol/L of 25-OH-D_3 showed marginal antiproliferative effect in PC-3 cells (Fig. 1*E*). We also observed that 10^{-6} mol/L of 25-OH-D_3-3-BE was cytotoxic only to LNCaP and PC-3 cells, causing the cells to lift, float, and die under phase contrast microscope.

In a cell counting assay, we observed that LNCaP and LAPC-4 cells had sharply reduced number with 10^{-6} mol/L of 25-OH-D_3-3-BE after 24 hours incubation (Fig. 2*A*), whereas MC3T3 cells were affected to a much lesser extent, and MCF-7 cells (incubated for 48 hours) were not significantly affected. It should be noted that in this assay cells were not serum starved before addition of the reagents, and 10^{-7} mol/L of 1,25$(OH)_2D_3$ had little effect in all of the cells. The 10^{-7} mol/L of 1,25$(OH)_2D_3$ was shown to produce significant effect in LNCaP cells after a longer period (3 to 6 days) of incubation (23). However, we observed that the effect of 10^{-6} mol/L of 25-OH-D_3-3-BE was relatively rapid (optimal antiproliferation and cytotoxicity in prostate cancer cells was observed within 16 to 24 hours of incubation). Therefore, within the short timeframe of our studies, 10^{-6} mol/L of 25-OH-D_3-3-BE produced a strong effect in LNCaP and LAPC-4 cells, whereas 10^{-7} mol/L of 1,25$(OH)_2D_3$ showed very little effect, if any, in all of the cells tested.

We conducted a dose-response study in which PC-3 cells were treated with 10^{-7} mol/L and 10^{-6} mol/L of either 25-OH-D_3-BE or 1,25$(OH)_2D_3$ for 18 hours followed by [^{3}H]thymidine incorporation assay. Results of this assay showed that 10^{-6} mol/L of 25-OH-D_3-BE decreased the proliferation of the cells

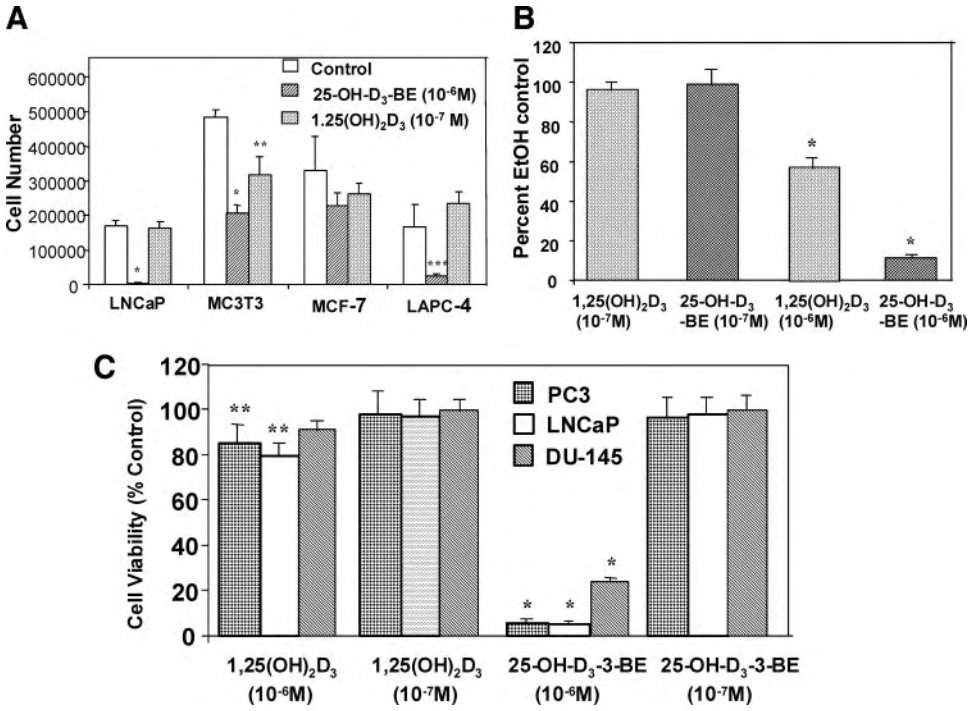

Fig. 2 *A*, cell counting assay of LAPC-4, LNCaP, MCF-7, and MC3T3 cells treated with 25-OH-D_3-3-BE or 1,25$(OH)_2D_3$. Each cell line was plated in 6-well plates at a density of either 80,000 cells per well (LNCaP, MCF-7, and MC3T3) or 160,000 cells per well (LAPC-4) and allowed to attach overnight. The cells were then treated for 24 hours (LNCaP, MC3T3, and LAPC-4) or 48 hours (MCF-7) with EtOH vehicle or 25-OH-D_3-3-BE (10^{-6} mol/L) or 1,25$(OH)_2D_3$ (10^{-7} mol/L). At the end of the experiment, cells were detached with trypsin-EDTA and counted in a Coulter counter. *, $P < 0.001$; **, $P = 0.003$; ***, $P = 0.014$. *B*, dose-response assays of 25-OH-D_3-3-BE or 1,25$(OH)_2D_3$ in PC-3 cells. Cells were incubated with either EtOH or 10^{-7} or 10^{-6} mol/L of 25-OH-D_3-3-BE or 1,25$(OH)_2D_3$ for 18 hours followed by [^{3}H]thymidine incorporation assay as described in Materials and Methods. *, $P < 0.005$. *C*, MTS cell viability assays of PC-3, LNCaP, and DU-145 cells. Cells were grown as described above and then treated with EtOH or 10^{-7} or 10^{-6} mol/L of either 25-OH-D_3-3-BE or 1,25$(OH)_2D_3$ for 20 hours followed by MTS assay as described in Materials and Methods, which included measurement of absorbance at 409 nm. Results are expressed in terms of percent of cell viability relative to EtOH-control (100%). *, $P < 0.0001$; **, $P < 0.05$. *Bars*, ±SD.

by ~90%, whereas there was ~45% reduction with 10^{-6} mol/L of $1,25(OH)_2D_3$. However, there was virtually no effect with 10^{-7} mol/L of either reagent (Fig. 2*B*). Furthermore, we observed that 25-OH-D_3-BE was toxic to these cells (as well as to LNCaP cells; Fig. 1*C*), as they were found detached and floating.

To elaborate on the cytotoxic nature of 25-OH-D_3-BE, we carried out MTS cell viability assay with LNCaP, PC-3, and DU-145 cells treated with 10^{-7} mol/L and 10^{-6} mol/L of 25-OH-D_3-BE or $1,25(OH)_2D_3$. Results of this assay (Fig. 2*C*) showed that 10^{-6} mol/L of 25-OH-D_3-BE reduced the number of viable cells to ~8% in LNCaP and PC-3 cells and 20% in DU-145 cells, whereas majority of the cells were viable when treated with 10^{-6} mol/L of $1,25(OH)_2D_3$. With 10^{-7} mol/L of 25-OH-D_3-BE and 10^{-7} mol/L and 10^{-6} mol/L of $1,25(OH)_2D_3$ majority of the cells were viable. These results suggested that 25-OH-D_3-BE induced toxicity in these cells at 10^{-6} mol/L. As mentioned earlier, repeated dosing of LNCaP cells with 10^{-7} mol/L of $1,25(OH)_2D_3$ for a prolonged period (48 hours) produced significant antiproliferative effect, whereas a single dose and shorter incubation period failed to produce such an effect (23). Therefore, it is probable that repeated dosing with 10^{-7} mol/L of 25-OH-D_3-BE for longer periods (we typically dosed the cells for 16 to 20 hours) might have produced significant antiproliferative and possibly cytotoxic effects.

Induction of toxicity in DU-145 cells deserves special attention, because it has been shown that DU-145 cells respond poorly to $1,25(OH)_2D_3$-treatment because of enhanced activity of the catabolic enzyme, 24-OHase (24, 25). We postulated that covalent attachment of 25-OH-D_3-BE into the ligand-binding pocket of VDR might prevent the catabolism of the analog and produce sufficient quantity of transcriptionally active VDR. Therefore, our results with DU-145 cells lend strong support for this hypothesis.

The growth inhibitory effect of $1,25(OH)_2D_3$ and its analogs is generally manifested via the arresting of cellular growth in G_0/G_1 phase; and such activity correlates well with the expression of cyclin-dependent kinase inhibitors, such as p21 and p27 (26). However, in some cases, apoptosis, or programmed cell death, has been reported. For example, it was reported that $1,25(OH)_2D_3$ induced apoptosis in MCF-7 cells (22, 27), although in prostate cancer cells reports are conflicting. For example, Blutt *et al.* (23) reported that $1,25(OH)_2D_3$ induced apoptosis in LNCaP cells, but another group failed to observe such an effect (28).

Fragmentation of nuclear DNA is a hallmark of the downstream process manifested by cells undergoing apoptosis. When PC-3 cells were treated with 0.25×10^{-6} mol/L of 25-OH-D_3-3-BE, 25-OH-D_3, or $1,25(OH)_2D_3$, DNA-fragmentation was observed only with cells treated with 25-OH-D_3-3-BE (Fig. 3*A*, *Lane 4*), whereas no such effect was visible with an equivalent amount of $1,25(OH)_2D_3$ (Fig. 3*A*, *Lane 2*) or 25-OH-D_3 (Fig. 3*A*, *Lane 3*). These results suggested that 25-OH-D_3-3-BE induced apoptosis in PC-3 cells, whereas an equivalent amount of 25-OH-D_3 and $1,25(OH)_2D_3$ failed to do so.

Caspases are key indicators of apoptosis in cells (29). For example, caspase 3 is activated during the cascade of events during apoptosis. It cleaves a variety of molecules containing DEVD amino acid motif. Such molecules include poly-ADP-ribose polymerase (PARP), U1-ribonucleoprotein, and so forth. Caspase 8 is an upstream caspase, and its activation leads to the activation of additional caspases and subsequent cleavage of PARP and other molecules. Caspase 9 is a key regulator of apoptosis *in vivo*. Activation of caspase 9 activates procaspase 3, which in turn is manifested through classical features of apoptosis such as cleavages of PARP, U1-ribonucleoprotein, and so forth.

Recently, Guzey *et al.* (30) reported that $1,25(OH)_2D_3$ activated caspase 3 and caspase 9, but not caspase 8, in ALVA-31 cells. Polek *et al.* (31) also showed that $1,25(OH)_2D_3$ did not induce apoptosis in PC-3 cells. When PC-3 cells were treated with 0.01×10^{-6} mol/L of 25-OH-D_3-3-BE or 25-OH-D_3 or $1,25(OH)_2D_3$, only 25-OH-D_3-3-BE showed strong induction of caspases 3, 8, and 9 (Fig. 3*B*). Therefore, DNA-fragmentation analysis and caspase-activation assay collectively suggested that 25-OH-D_3-3-BE induced apoptosis in PC-3 cells.

The 25-OH-D_3-3-BE contains an ester bond. Therefore, esterases in growing cells might hydrolyze this molecule to

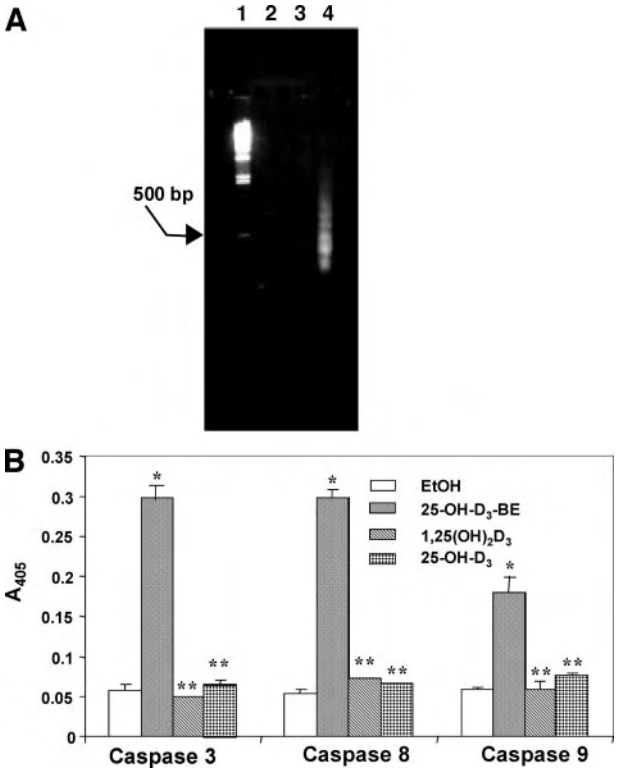

Fig. 3 *A*, DNA fragmentation analysis of PC-3 cells treated with 25-OH-D_3-3-BE or $1,25(OH)_2D_3$ or 25-OH-D_3. Cells were treated with 0.25×10^{-6} mol/L of reagents for 20 hours followed by extraction of the DNA of the cells undergoing apoptosis, and analysis on a DNA gel. The gel was visualized by ethidinium bromide staining. *Lane 1*, DNA markers; *Lane 2*, $1,25(OH)_2D_3$; *Lane 3*, 25-OH-D_3; *Lane 4*, 25-OH-D_3-3-BE. It should be noted that under the condition of our experiment, only the apoptotic DNA fragments leaking into the cytosol were extracted. *B*, caspase activation analysis of PC-3 cells treated with 25-OH-D_3-3-BE or $1,25(OH)_2D_3$ or 25-OH-D_3. Cells were incubated with 0.01×10^{-6} mol/L of 25-OH-D_3-3-BE, $1,25(OH)_2D_3$ or 25-OH-D_3 followed by colorimetric assays for caspase 3, 8, and 9 according to manufacturer's procedures. The X-axis represents absorbance of the solutions at 405 nm. *, $P < 0.0035$. *Bars*, ±SD.

produce equimolar amounts of 25-OH-D_3 and bromoacetic acid (Fig. 4, *top panel*). It could be argued that the observed effects of 25-OH-D_3-3-BE might be because of bromoacetic acid, 25-OH-D_3, or a combination of the two. To determine any role of *in situ*-produced bromoacetic acid (by the hydrolysis of 25-OH-D_3-3-BE), we carried out [^{3}H]thymidine incorporation assay in PC-3 cells treated with 10^{-6} mol/L of either bromoacetic acid or 25-OH-D_3-3-BE or a mixture containing 10^{-6} mol/L each of bromoacetic acid or 25-OH-D_3-3-BE. As shown in Fig. 4 (*bottom left panel*), 10^{-6} mol/L of 25-OH-D_3-3-BE was strongly antiproliferative to the cells, whereas 10^{-6} mol/L of bromoacetic acid did not have any significant effect on the proliferation of these cells. Furthermore, a mixture containing 10^{-6} mol/L each of bromoacetic acid and 25-OH-D_3-3-BE produced the same effects as 10^{-6} mol/L of 25-OH-D_3-3-BE alone (Fig. 4, *bottom right panel*). Therefore, these results strongly suggested that the observed properties of 25-OH-D_3-3-BE were related to its unhydrolyzed (intact) form.

However, the above results did not rule out the possibility that a part of 25-OH-D_3-3-BE might undergo hydrolysis, and 25-OH-D_3, produced *in situ* by this hydrolytic process, might be metabolically activated by 25-hydroxyvitamin D_3-1α-hydroxylase (1-OHase) to 1,25$(OH)_2D_3$, which could in turn produce the observed effects, at least partially. LNCaP cells are known to be deficient in the 1-OHase enzyme (32), yet 25-OH-D_3-3-BE showed strong antiproliferative effect in these cells (Fig. 1*C*). Furthermore, 10^{-6} mol/L of 25-OH-D_3 showed a very weak effect in PC-3 cells (Fig. 1*E*). These considerations essentially ruled out any role of *in situ*-produced 25-OH-D_3 in the observed antiproliferative and cytotoxic properties of 25-OH-D_3-3-BE.

The 25-OH-D_3-3-BE contains a chemically reactive α-halocarbonyl group; therefore, it could potentially alkylate any protein in a cellular system, and such random interaction could possibly be responsible for its observed effects. FBS contains many proteins, including a relatively large amount of vitamin D-binding protein, which could potentially react with 25-OH-D_3-3-BE, and eliminate it completely before it reacts with VDR. Typically, the assays described here were carried out in a media containing 5 to 10% FBS, suggesting that scavenging of 25-OH-D_3-3-BE by serum vitamin D-binding protein (and other cellular proteins in a random fashion) might not play a significant role in the observed properties of this compound.

Because VDR was our desired target to elicit the biological activity of 25-OH-D_3-3-BE, it became obligatory for us to show the involvement of processes related to 1,25$(OH)_2D_3$/VDR-signaling pathways. The 24-OHase gene is known to be strongly and predictably modulated by 1,25$(OH)_2D_3$ and its analogs. We carried out a study to evaluate the effect of 1,25$(OH)_2D_3$ and 25-OH-D_3-3-BE at various doses on the 24-OHase promoter activity in COS-7 cells that was transfected with a VDR construct, tagged with a CAT reporter gene. Results of this assay, shown in Fig. 5, showed that 24-OHase promoter activity was strongly up-regulated by 10^{-8}, 10^{-7}, and 10^{-6} mol/L of 1,25$(OH)_2D_3$. In contrast, strong promoter activity was displayed only with 10^{-6} mol/L of 25-OH-D_3-3-BE, and such activity declined almost to the basal level with 10^{-7} mol/L of 25-OH-D_3-3-BE. These results strongly suggested that the molecular action of 25-OH-D_3-3-BE might follow a path similar to 1,25$(OH)_2D_3$, however, with less efficiency.

An important aspect of ligand-receptor interaction is the ability of the hormone and the analogs to induce transcriptionally active conformation in VDR that can interact with RXR and other coactivators required for transcription, such as GRIP-1 (33). Therefore, to determine the potency of 1,25$(OH)_2D_3$ and 25-OH-D_3-3-BE to induce interaction of VDR with RXR and/or with the steroid receptor coactivator, GRIP-1, we used a pull

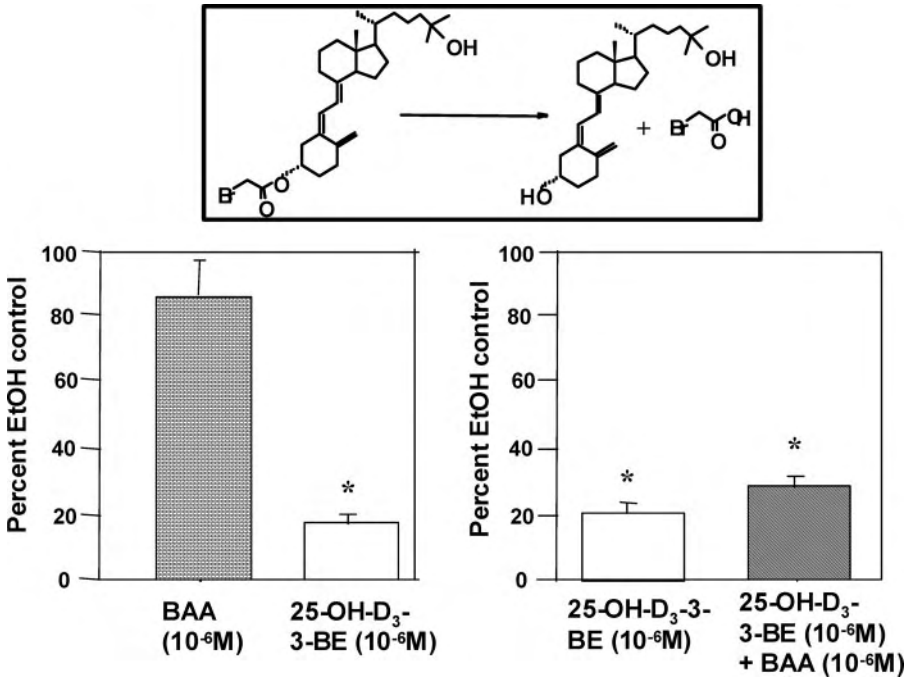

Fig. 4 top panel, scheme to show probable hydrolysis of the ester bond in 25-OH-D_3-3-BE to produce equimolar quantities of 25-OH-D_3 and bromoacetic acid. *bottom panel*, effects of 10^{-6} mol/L of 25-OH-D_3-3-BE or 1,25$(OH)_2D_3$ or bromoacetic acid or a combination of 25-OH-D_3-3-BE and bromoacetic acid (10^{-6} mol/L each) on the proliferation of PC-3 cells. Cells were treated with EtOH or 10^{-6} mol/L of the reagents and subjected to [^{3}H]thymidine incorporation assay in the usual fashion. *, $P < 0.005$. *Bars*, ±SD. (*BAA*, bromoacetic acid)

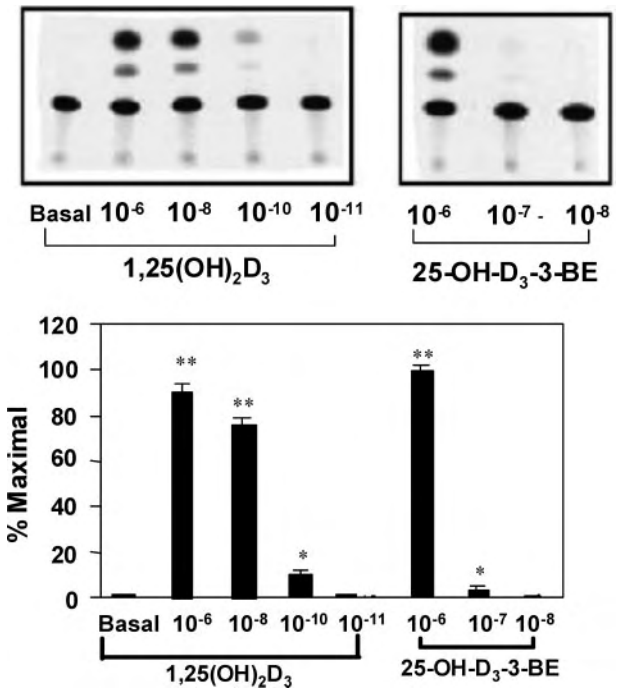

Fig. 5 Analysis of 24-OHase promoter activity in COS-7 cells, transiently transfected with a 24-OHase-construct, tagged with a chloramphenicon (CAT) reporter gene, and hVDR expression vector. Cells were treated with various doses (as indicated) of 25-OH-D_3-3-BE or $1,25(OH)_2D_3$, and CAT activity was determined as described in Materials and Methods. *% Maximal* in the X-axis denotes percentage of maximum activity (in this case with 10^{-6} mol/L of 25-OH-D_3-3-BE). *, $P < 0.005$; **, $P < 0.0001$. *Bars*, ±SD.

down assay in which PC-3 cells were incubated with various doses of $1,25(OH)_2D_3$ or 25-OH-D_3-3-BE, and VDR-interacting proteins were pulled down with GST-fused GRIP or RXR. Results of these assays showed strong interaction between VDR and GRIP-1 when the cells were incubated for 1 hour with 25-OH-D_3-3-BE (10^{-6} mol/L) or $1,25(OH)_2D_3$ (10^{-7} mol/L; Fig. 6, *left panel*). However, after 24 hours of incubation, strong interaction between VDR and GRIP-1 was observed with 10^{-7} mol/L of 25-OH-D_3-3-BE. With RXR, there was significant interaction even with 10^{-8} mol/L of 25-OH-D_3-3-BE (Fig. 6, *right panel*).

The above results provided the evidence that 25-OH-D_3-3-BE was able to activate VDR at substantially lower concentrations in PC-3 cells; which is consistent with the results of DNA-fragmentation and caspase-activation analysis. However, a significantly higher dose (10^{-6} mol/L) of 25-OH-D_3-3-BE was required to show 24-OHase–promoter activity in COS cells as well as antiproliferative activity in various cells. These discrepancies underscore the hypothesis that gene regulatory events leading to inhibition of cell growth might be different from those leading to apoptosis. Whether or not all of these cellular events are mediated through transcriptional activity of the VDR remains to be established. Furthermore, differences in the potency of analogs to induce different gene regulatory events through VDR in the same cell type have been reported by several studies, including Shevde *et al.* (34). This study with 2MD, an analog of $1,25(OH)_2D_3$, showed a range of sensitivity for regulating gene expression, from $ED_{50} = 10^{-11}$ mol/L for the up-regulation of RANKL to $ED_{50} > 10^{-10}$ mol/L for induction of the VDR responsive genes, osteopontin and 24-hydroxylase in mouse osteoblasts. Likewise, Ismail *et al.* (35) showed that the analog Ro-26-9228 had an ED_{50} of 2.1×10^{-8} mol/L for the induction of 24-OHase and an ED_{50} of 2.65×10^{-7} mol/L for induction of Calbindin D9k in Caco-2 cells.

A major concern involving $1,25(OH)_2D_3$ and its analogs is systemic toxicity (hypercalcemia, hypercalciuria) that is often found to be associated with these molecules. Therefore, if 25-OH-D_3-3-BE and related compounds were to be developed as therapeutic agents, they should be devoid of systemic toxicity. Although it is difficult to draw a direct correlation between *in vitro* and *in vivo* dosages, it was clear that doses (of 25-OH-D_3-BE) that might be required to reach a potential therapeutic level would be significantly higher than what has been customary

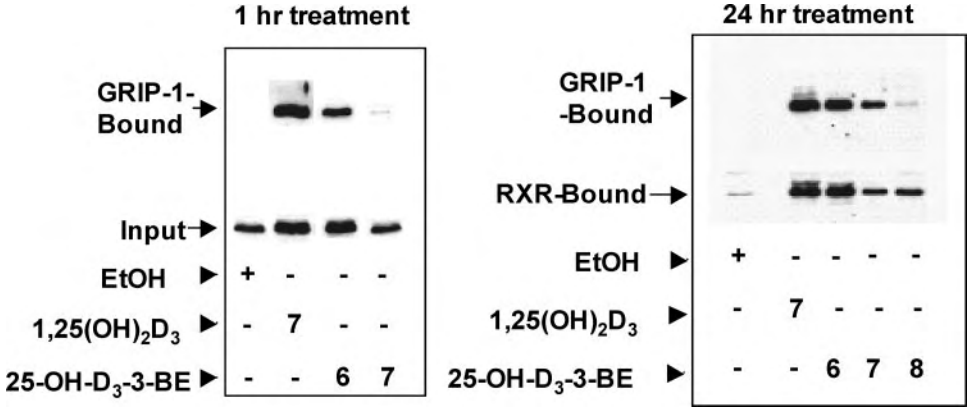

Fig. 6 Pull down assays in PC-3 cells to determine the interaction between VDR and RXR and GRIP-1, as the cells were treated with various doses of 25-OH-D_3-3-BE or $1,25(OH)_2D_3$ followed by incubation with GST-RXR or GST-GRIP-1 fusion proteins. The RXR and GRIP-1 bound proteins were adsorbed on glutathione-Sepharose beads. The bound proteins were eluted from the beads by boiling in Laemmli buffer and were analyzed by polyacrylamide gel electrophoresis. The protein bands were transferred onto a polyvinylidene difluoride membrane, and blots were visualized by Western blotting with an anti-VDR antibody.

Table 1 Body weight and serum calcium value of CD-1 mice treated with various doses of 25-OH-D_3 or 25-OH-D_3-3-BE

	Body weight on day 1 (grams)	Body weight on day 12 (grams)	Serum calcium on day 12 (mg/mL)
Saline-EtOH control	29.6 ± 2.4	29.6 ± 2.4	9.23 ± 0.15
25-OH-D_3-3-BE (3.3 μg/kg)	29.0 ± 0.6	29.0 ± 0.6	9.10 ± 0.20
25-OH-D_3-3-BE (33 μg/kg)	31.0 ± 0.83	32.9 ± 1.4	9.4 ± 0.20
25-OH-D_3-3-BE (166.7 μg/kg)	30.55 ± 0.7	30.7 ± 1.0	9.7 ± 0.10
25-OH-D_3 (3.3 μg/kg)	31.5 ± 1.56	33.5 ± 1	9.6 ± 0.2
25-OH-D_3 (33 μg/kg)	28.9 ± 2	30.66 ± 2	9.7 ± 0.8

with 1,25$(OH)_2D_3$ and its analogs. However, we surmised that an analog of 25-OH-D_3/1,25$(OH)_2D_3$ could be useful in higher concentrations as long as it did not show systemic toxicity. For example, higher than customary doses of 1α-hydroxyvitamin D_5 were used *in vivo* to elicit desired effects (36).

We carried out a toxicity study of 25-OH-D_3-BE in CD-1 mice where we used 25-OH-D_3 as a control. Our purpose was to determine whether we could extrapolate the nontoxic property of 25-OH-D_3 to its analog (*i.e.*, 25-OH-D_3-BE) and to obtain a preliminary idea about the safe dose levels of 25-OH-D_3-BE. As shown by the results (Table 1), there was no significant difference in serum calcium values and weights of the animals from the vehicle control with 3.3 or 33.0 μg/kg of 25-OH-D_3 or 25-OH-D_3-BE. Although there was a slight increase in serum calcium value only with the highest dose (166.7 μg/kg) of 25-OH-D_3-BE, body weights of the animals were not significantly different from the vehicle control. It should be emphasized that the above results simply denoted that 25-OH-D_3-BE had a significantly lower toxicity than 1,25$(OH)_2D_3$ or majority of its analogs without providing any information on its effective serum concentration and bioavailability. We have shown that 25-OH-D_3-BE is the active molecule that is responsible for the observed antiproliferative activity in prostate cancer cells (Fig. 4). But, we appreciate that 25-OH-D_3-BE can undergo hydrolytic cleavage *in vivo* to reduce its bioavailability. In the future, we will carry out pharmacokinetic and pharmacodynamic studies to shed light on this issue.

In toxicity studies, it is customary to use 1,25$(OH)_2D_3$ as a control. But 1,25$(OH)_2D_3$ and many of its synthetic analogs are known to be toxic at doses used in our study. For example, in a recent publication it was reported that 1.0 μg/kg of 1,25$(OH)_2D_3$ and EB-1089 [a noncalcemic analog of 1,25$(OH)_2D_3$] raised serum calcium beyond vehicle control, although significantly less with EB-1089 than 1,25$(OH)_2D_3$ (37). For obvious reasons, we could not use 1,25$(OH)_2D_3$ or EB-1089 as controls at high dose levels that were used in our toxicity study with 25-OH-D_3-BE.

The 1,25$(OH)_2D_3$ and its analogs are generally not known to have tissue/tumor specific effects because of the ubiquitous nature of VDR, the chief modulator of their actions. In this communication, we report that 25-OH-D_3-3-BE, a VDR-affinity alkylating derivative of the prehormone, displayed strong antiproliferative activity in androgen-sensitive LNCaP and LAPC-4 and androgen refractory PC-3 and DU-145 cells. In addition, 25-OH-D_3-3-BE induced apoptosis in these prostate cancer cells but not in normal immortalized prostate cells (PZ-HPV-7) at the same dose level. The reason behind the prostate cancer cell-specific effects of 25-OH-D_3-3-BE can only be speculated at this point. It is noteworthy that 1,25$(OH)_2D_3$-3-BE, the 1,25$(OH)_2D_3$ prototype of 25-OH-D_3-3-BE, showed very similar antiproliferative and apoptotic behavior (as 25-OH-D_3-3-BE) in prostate cancer cells (17). Therefore, we surmise that covalent labeling of the hormone binding pocket [by 25-OH-D_3-3-BE and 1,25$(OH)_2D_3$-3-BE] is crucial for their prostate cancer-specific effects. However, antiproliferative index of C-1 and C-11 bromoacetates of 1,25$(OH)_2D_3$ [which affinity alkylated VDR similar to 1,25$(OH)_2D_3$-3-BE] in keratinocytes was much lower than 1,25$(OH)_2D_3$-3-BE.[6] This suggested that covalent modification of a specific area of VDR [by 3-boromoactetates: 25-OH-D_3-3-BE and 1,25$(OH)_2D_3$-3-BE] has a profound effect on transcriptional activities. We postulate that 25-OH-D_3-3-BE changes the conformation of VDR (on alkylation) so that the liganded receptor specifically and uniquely modulate certain factor/factors directly or indirectly in the prostate cancer cells. We are currently in the process of identifying such factor/factors by gene-profiling experiments.

Several clinical trials involving 1,25$(OH)_2D_3$ and its analogs in prostate and other cancers are currently underway. Results of the studies described in this report strongly suggest that 25-OH-D_3-3-BE and related VDR-cross linking analogs of 25-OH-D_3 might be useful as potential therapeutic agents for androgen-sensitive and androgen-refractory prostate cancer.

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Growth inhibition of carcinogen-transformed MCF-12F breast epithelial cells and hormone-sensitive BT-474 breast cancer cells by 1α-hydroxyvitamin D5

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Several studies have established the active form of vitamin D_3 as an effective tumor-suppressing agent; however, its antitumor activity is achieved at doses that are hypercalcemic *in vivo*. Therefore, less calcemic vitamin D_3 analog, 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5), was evaluated for its potential use in breast cancer chemoprevention. Previously, 1α(OH)D5 showed anticarcinogenic activity in several *in vivo* and *in vitro* models. However, its effects on growth of normal tissue were not known. The present study was conducted to determine the effects of 1α(OH)D5 on the growth of normal mouse mammary gland and normal-like human breast epithelial MCF-12F cells and to compare these effects with carcinogen-transformed MCF-12F and breast cancer cells. No significant difference was observed in the growth or morphology of cultured mouse mammary gland and MCF-12F cells in the presence of 1α(OH)D5. However, the transformed MCF-12F cells underwent growth inhibition (40–60%, $P < 0.05$) upon 1α(OH)D5 treatment as determined by cell viability assays. Cell cycle analysis showed marked increase (50%) in G-1 phase for cells treated with 1α(OH)D5 compared with the controls. Moreover, the percentage of cells in the synthesis (S) phase of cell cycle was decreased by 70% in transformed MCF-12F, BT-474 and MCF-7 cells. The growth arrest was preceded by an increase in expression of cell cycle regulatory proteins $p21^{Waf-1}$ and $p27^{Kip-1}$. In addition, differential expression studies of parent and transformed MCF-12F cell lines using microarrays showed that prohibitin mRNA was increased 4-fold in the transformed cells. These results indicate that the growth inhibitory effect of 1α(OH)D5 was achieved in both carcinogen-transformed MCF-12F and breast cancer cells at a dose that was non-inhibitory in normal-like breast epithelial cells.

Introduction

Cancer is a multistage process that involves transformation of normal tissue to malignant phenotype. Breast cancer is the second leading cause of cancer-related deaths among women in the USA. Since breast cancer results in considerable morbidity and patient care cost, chemopreventive strategies for breast cancer management are being extensively studied. Chemoprevention implies intake of one or several compounds, either natural or synthetic, to block or delay the onset or progression of cancer (1,2). Ideally, a chemopreventive agent should promote cell differentiation and selective growth inhibition of potentially malignant cells. Several epidemiological studies have supported the negative association of breast, prostate and colon cancer mortality rates with annual sunlight exposure and serum vitamin D_3 levels in different geographical areas (3–5). Based on these observations, the chemopreventive potential of vitamin D_3 has been explored in various *in vivo* as well as *in vitro* models.

Vitamin D_3 is a seco-steroid, which is classified as a fat-soluble vitamin. Its dietary intake is not crucial as it can be synthesized in the skin with sunlight exposure. It has to undergo two hydroxylation steps to attain biological activity. The active form of vitamin D_3, 1α,25$(OH)_2D_3$ or calcitriol, has two major biological functions: (i) regulation of calcium and phosphorus homeostasis and metabolism and (ii) induction of cell differentiation and growth modulation (6). Calcitriol exerts its biological activity through both genomic and non-genomic pathways; the genomic pathway involves signaling through a high affinity nuclear receptor (vitamin D_3 receptor or VDR). Calcitriol is now well recognized as an effective growth suppressing and differentiating agent for leukemia, melanoma, breast, colon and prostate cancer cells *in vitro* (7,8). However, the antiproliferative effects of 1α,25$(OH)_2D_3$ are achieved at concentrations that are hypercalcemic and toxic *in vivo*. Consequently, much attention has been paid to developing less calcemic and safer analogs of 1α,25$(OH)_2D_3$ (referred to as deltanoids) that would have desirable growth modulatory effects. Over 2000 different deltanoids have been synthesized so far, with most containing structural changes in the C or D ring, or the side chain of vitamin D_3 molecule. These deltanoids are designed to be selective VDR agonists. Out of the nearly 2000 synthetic deltanoids, only a few have shown potential for use in chemoprevention or therapy of breast cancer.

An analog of 1α,25$(OH)_2D_3$, 1α-hydroxy-24-ethyl-cholecalciferol (1α(OH)D5), has been evaluated for the past few years for its potential use in breast cancer chemoprevention (9,10). It has been shown to be one of the least toxic of all the vitamin D series of compounds tested (11). Previous studies have shown that 1α(OH)D5 at 1 μM concentration inhibited development of carcinogen-induced precancerous lesions in mouse mammary gland organ culture (9). In rodent models, 1α(OH)D5 supplemented diet reduced the incidence and multiplicity of mammary tumors. Furthermore, 1α(OH)D5 supplied in the diet increased the latency period for N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis in rats (12). Specifically, the effect of 1α(OH)D5 in dimethylbenz(a)anthracene (DMBA)-induced rat mammary

Abbreviations: 1α[OH]D5, 1α-hydroxy-24-ethyl-cholecalciferol; ER, estrogen receptor; DMBA, dimethylbenz(a)anthracene; MNU, N-methyl-N-nitrosourea; MTT, 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; VDR, vitamin D_3 receptor; VDRKO, VDR knockout.

carcinogenesis model was selective during the promotion phase of carcinogenesis (10). In addition, in a xenograft mouse model, 1α(OH)D5 inhibited the growth of VDR-positive BT-474 cancer breast cells (13).

Although previous studies have established the growth inhibitory action of 1α(OH)D5 on cancer tissue both *in vivo* and *in vitro*, no data on its effect on growth of normal or normal-like breast epithelial cells were available. Therefore, the present study aimed to compare the growth effects of 1α(OH)D5 on normal, transformed and cancer breast epithelial cells. Additionally, this study examines the effect of 1α(OH)D5 on the expression of various markers of cell proliferation and cell cycle regulation.

Materials and methods

Vitamin D_3 analog, 1α(OH)D5

1α(OH)D5 was synthesized according to the procedure described previously (9). The stock solution of 10 mM in ethanol was stored at −80°C. 1α(OH)D5 was diluted in ethanol to a concentration of 1 mM for working solution. The proportion of ethanol was <0.01% of the total cell culture media. The appropriate controls for each experiment consisted of treatment with the vehicle ethanol. All reagents were purchased from Sigma-Aldrich (St Louis, MO).

Mouse mammary gland organ culture

In order to determine the growth effects of 1α(OH)D5 on normal mammary glands, Balb/c mice were killed to obtain mammary glands according to the protocol described previously (9). Briefly, mammary glands from 4-week-old female virgin Balb/c mice were divided into six groups with 15 glands per group: control, 1α,25$(OH)_2D_3$ (0.1 μM), and 1α(OH)D5-treated (1 μM) for alveolar growth; control, 1α,25$(OH)_2D_3$ (0.1 μM), and 1α(OH)D5-treated (1 μM) for ductal growth. The glands were cultured in the presence of aldosterone (1 μg/ml) and hydrocortisone (1 μg/ml) for the growth of alveolar structures, whereas progesterone (1 μg/ml) and 17β-estradiol (0.001 μg/ml) were used for inducing growth of ductal structures. The glands were treated for 6 days and then stained and compared for the morphological differences between the controls and the 1α(OH)D5-treated glands, with 1α,25$(OH)_2D_3$ as a positive control. In an earlier report, 1α,25$(OH)_2D_3$ exhibited maximum growth inhibition of lesion formation at 0.1 μM; whereas, 1α(OH)D5 was required at 1 μM concentration to get the maximum efficacy. At higher concentrations 1α,25$(OH)_2D_3$ was toxic and 1α(OH)D5 did not have any additional suppression of the lesion development (9). Therefore, for the present studies, 0.1 μM and 1 μM of 1α,25$(OH)_2D_3$ and 1α(OH)D5 were used, respectively. The histopathological sections were compared for ductal proliferation of glands.

Breast epithelial and cancer cell cultures

Normal-like MCF-12F cells, which are derived from reduction mammoplasty, were used in the present study. These cells were transformed with carcinogens to obtain the cell lines MCF-12F_{MNU} and MCF-12F_{DMBA} as described in the following section. The breast cancer cells used for the present study included MCF-7 and BT-474 (steroid receptor positive) and MDA-MB-231 and MDA-MB-435 (steroid receptor negative) cells. All the cell lines express VDR in varying proportions.

Human breast epithelial cells MCF-12F were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified essential media with Ham's F-12 nutrient mix (DMEM/F12) containing 5% Chelex-treated horse serum. The tissue culture medium also contained epidermal growth factor (EGF, 10 μg/500 ml), cholera toxin (50 μg/500 ml), insulin (5 mg/500 ml) and hydrocortisone (250 μg/500 ml) along with antibiotic and antimycotic reagents. The transformed MCF-12F cells were cultured in similar media with the exception of horse serum—instead, the heat-inactivated fetal bovine serum (FBS) was used. The human breast cancer cells BT-474, MCF-7, MDA-MB-231 and MDA-MB-435 were purchased from ATCC and maintained according to the ATCC directions. All breast cancer cells were maintained in Minimum Essential Medium with Earle's salts (MEME) fortified with a mixture of non-essential amino acids and 5% FBS. For MCF-7 cells, insulin (2 μg/ml), sodium pyruvate (0.01 mM) and sodium bicarbonate (0.15%) were also added. All experiments on these cells were performed in the regular culture conditions and all cell culture chemicals were purchased from Invitrogen (Carlsbad, CA).

Carcinogen-induced transformation of MCF-12F cells

The spontaneously immortalized breast epithelial cells MCF-12F are useful in understanding the effects of chemopreventive agents in normal tissue, but do not provide insight into the effects on preneoplastic tissues (14). In order to compare the growth effects of 1α(OH)D5 on transformed cells with that on normal-like MCF-12F cells, the MCF-12F cells were transformed using two different mammary specific carcinogens. Once the two transformed MCF-12F_{DMBA} and MCF-12F_{MNU} cell lines were established, their growth characteristics were compared with MCF-12F cells using phase contrast microscopy for morphological examination, Boyden Chamber assay for invasive potential and cell growth studies for the rate of proliferation.

Carcinogen treatment was used for the transformation of MCF-12F cells as described previously (15). The two mammary specific carcinogens, DMBA and MNU were used. The MCF-12F cells (passage 12) were grown to subconfluency in a cell culture dish and the cells were incubated with DMBA dissolved in DMSO (2 μg/ml) for 24 h followed by another 24 h incubation with fresh DMBA in the media. This resulted in extensive cell death. The surviving cells were washed with phosphate buffered saline (PBS) and allowed to grow in fresh media without DMBA until confluent. The cells were subcultured and then gradually serum starved and then brought back to 5% FBS. The remaining cells were allowed to grow in regular media and the cell line MCF-12F_{DMBA} was established. For MNU-induced transformation, the carcinogen was dissolved in acidified saline (pH 5.3 with acetic acid) and used within 20 min of preparation. MCF-12F cells (passage 12) were exposed to MNU (2 μg/ml) twice daily for 2 days. The cell line MCF-12F_{MNU} was similarly established.

Boyden chamber assay

Boyden Chamber Assay was used to determine the invasive potential of breast epithelial cells through a Matrigel® coated membrane (16). The Boyden chambers (BioCoat Becton-Dickinson, BD Biosciences, Palo Alto, CA) consisted of a 12-well cell culture plate with Matrigel® coated inserts that were made up of polycarbonate membrane (pore size 8 μm and surface diameter 6.5 mm). All MCF-12F cells were plated in the cell culture plate with Matrigel® inserts and treated with 1α(OH)D5 or control. After 48 h of treatment, the incubation was terminated and the tops of the membranes were scraped to remove the plated cells. The membranes were then stained with Gills no.1 hematoxylin. The number of cells on the bottom of membranes was counted to determine the relative percentage of cells that invaded through the Matrigel® inserts. Each experiment was conducted in triplicates and at least five fields were counted for each insert using 20× magnification. The results were reported as the percentage of control.

Cell growth studies

Cell growth studies were performed using cell count with Coulter Counter® Cell and Particle Analyzer to determine the difference in the cell number between the treated and the control groups. In addition to the cell count, measurement of cell proliferation based upon the reduction of the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was used to assess the cell viability (17). MTT assays were performed using 96-well culture plates; 16 wells per group were used and the data were reported as mean and SEM after adjustment for control.

Cell cycle analysis

To further understand the nature of growth inhibitory effects of 1α(OH)D5 on breast epithelial cells, cell cycle analysis was conducted using DNA content measurement via flow cytometry (18). Briefly, after trypsinization, the cell pellets were resuspended and fixed using ice-cold ethanol. The fixed cells were then resuspended in citrate buffer (containing 40 mM trisodium citrate and 250 mM sucrose) and incubated with propidium iodide stain and spermine tetrahydrochloride to stabilize the nuclei. The DNA content analysis was performed on a Beckman Coulter EPICS Elite Flow Cytometer (Beckman Coulter, Fullerton, CA) and cell cycle parameters based upon DNA content were calculated using the EXPO32 Flow Cytometry software (Beckman Coulter). The data were expressed as the percentage of cell population in G-1, S and G-2 phases of the cell cycle. Samples were analyzed in triplicates and data were expressed as mean and SEM.

Expression studies

To determine whether 1α(OH)D5 treatment affects the cell cycle-related protein expression, BT-474 cells were treated with 1α(OH)D5 or the control for various time points. Total soluble proteins and total RNA were extracted and subjected to western blot and RT–PCR analyses, respectively. For western blots, the primary antibodies were purchased from Neomarkers (Lab Vision, Fremont, CA). All expression studies were repeated at least twice and the data were reported as percentage increase or decrease relative to the respective controls after adjustment for the housekeeping gene β-actin. The protein and

RT–PCR product bands were photographed using the Kodak 1D Image Analysis Software version 3.5 (Kodak Digital Science Imaging, Eastman Kodak Company, New Haven, CT) and analyzed to compare the model net intensity of each band.

Proteins were extracted from control and treated cells using a protein lysis buffer containing HEPES (pH 7.9, 20 mM), NaCl (400 mM), Nonidet P-40 (0.1%), Glycerol (10%), Na Vanadate (1 mM) and Na Fluoride (1 mM) along with a cocktail of protease inhibitors. The soluble proteins were stored at −20°C until separation by electrophoresis. The concentration of each sample was determined by using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) and 50 μg proteins were loaded in each well. Protein separation was performed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (19). The proteins were transferred to a nitrocellulose membrane (Fisher Scientific, Pittsburgh, PA). Towbin buffer with 20% methanol was used in an electro-blotting tank for the transfer of proteins from the gel onto the membrane. After the transfer was completed, the membrane was probed with primary antibody in 2% skim milk dissolved in tris-buffered saline with Tween-20 (TBST; 10 mM tris-HCl, 150 mM NaCl and 0.05% Tween-20) for 2 h at room temperature or overnight at 4°C. The membrane was washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30–45 min followed by TBST rinses. Finally, the electrochemiluminescent (ECL, Amersham Biosciences, Piscataway, NJ) reagent was used to visualize the proteins on the photographic film (Kodak X-Omat film, Fisher Scientific, Pittsburgh, PA).

For RT–PCR, total RNA was extracted using Clontech's Nucleospin RNA II kit (Clontech, BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. RT–PCR was performed with 0.5 μg of the total RNA from control and treated samples using the Superscript™ One-Step RT–PCR system with Platinum® Taq (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Gene-specific primers for $p21^{Waf\text{-}1}$ were 5′-TGAAGGCAGGGGGAAGGT-3′ and 5′-AGTATGTTACAGGAGCTGGA-3′ yielding a 762 bp fragment, while for $p27^{Kip\text{-}1}$ a 934 bp fragment was amplified using 5′-TAGAGCTCGGGCCGTGGCT-3′ and 5′-GTCCATTCCATGAAGTCAGCGATATG-3′ primers. The amplified product was kept refrigerated until run on an agarose (1%) gel containing ethidium bromide for UV visualization of DNA. In addition, RT–PCR was performed on RNA extracts from control and treated MCF-12F cell lines to determine the expression of prohibitin. The primers used for prohibitin amplification were 5′-ACCACGTAATGTGCCAGTCA-3′ and 5′-CCGCTTCTGTGAACTCCTTC-3′, yielding a 332 bp fragment.

Microarray analysis

Comparisons of the differential gene expression between the mRNA derived from control and treated MCF-12F and MCF-12F_{MNU} cell extracts were performed by the Clontech, (http://atlas.clontech.com/) using human 8K Atlas array. The RNA quality and integrity were evaluated by Clontech prior to the analyses. The samples were submitted without revealing the identity to the Clontech. In the first analysis, MCF-12F RNA extracts were compared with the MCF-12F_{MNU} extracts. Whereas, the second analysis was a comparison of the control and 1α(OH)D5-treated MCF-12F_{MNU} cell extracts. Each analysis was run in duplicates and the data were normalized using the global sum method with a coefficient of 1.35 (Clontech).

Data analysis

Cell growth data including cell count, MTT assay and cell cycle analysis were expressed as mean and SEM with *P*-values obtained via the student's *t*-test (two-way, Type I error $\alpha = 0.05$) using Instat® version 3.0 (GraphPad Software, San Diego, CA). All other data were expressed as the percentage of control. Microsoft® Excel 2000 was used to graphically plot all data. Kodak 1D Image Analysis Software version 3.5 (Kodak Digital Science Imaging, Eastman Kodak Company, New Haven, CT) was used to determine the band density on western blot films for protein expression and on agarose gels for RT–PCR products. The band density data were standardized with the housekeeping gene β-actin. After being adjusted for appropriate controls, the data were plotted using *XY* scatter plot. The trend lines were generated using polynomial regression analysis (Prism® version 2.01, GraphPad Software, San Diego, CA).

Results

Effects of 1α(OH)D5 on the growth and proliferation of normal mouse mammary glands and normal-like human breast epithelial (MCF-12F) cells.

In order to determine the effects of 1α(OH)D5 on the growth and morphology of normal mammary glands, normal mammary glands from female Balb-c mice were incubated with growth promoting hormones for 6 days in the presence or absence of 1α(OH)D5 (1 μM). There was no difference observed between the morphology of the control glands and the glands treated with 1α(OH)D5 (Figure 1). Neither the alveolar nor the ductal growth was affected by the presence of 1 μM 1α(OH)D5 in the normal mammary gland culture.

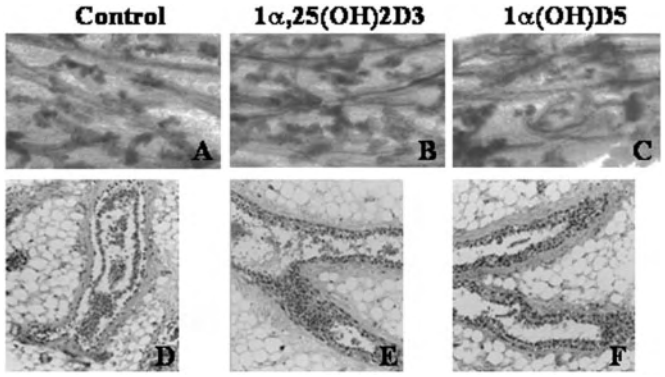

Fig. 1. Mouse mammary glands from Balb/c mice were incubated with growth promoting hormones in serum-free medium for 6 days with or without 1α(OH)D5. (**A**–**C**), Whole mounts of the glands were stained with carmine and analyzed for alveolar growth. (**D**–**F**), Glands were fixed, paraffin embedded, and stained with hematoxylin and eosin to observe the ductal growth. (**A**) and (**D**), Control; (**B**) and (**E**) 1α,25$(OH)_2D_3$ (0.1 μM); and (**C**) and (**F**) 1α(OH)D5 (1 μM).

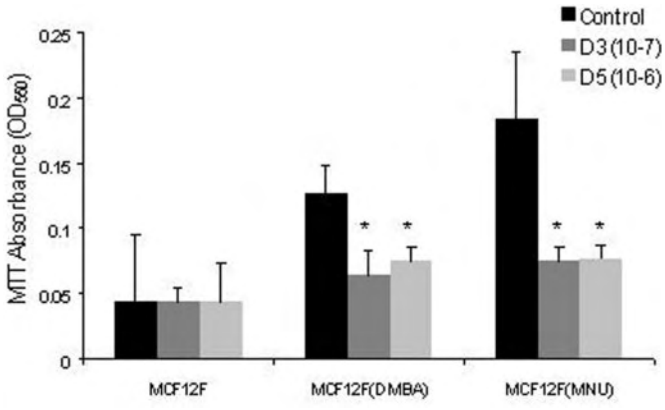

Fig. 2. Effect of 1α(OH)D5 on the growth of MCF-12F, MCF-12F_{DMBA} and MCF-12F_{MNU} cells. The cells were tested for viability using MTT absorbance assay 3 days after 1α(OH)D5 (1 μM) treatment. $^{*}P$-value <0.05. Mean (± SEM) were compared using two-tailed *t*-tests.

Normal-like breast epithelial cells, MCF-12F, were treated with different doses of 1α(OH)D5 (0.1–5 μM) as well as with a non-toxic (0.1 μM) dose of 1α,25$(OH)_2D_3$. No significant growth inhibition was observed upon treatment with 1α(OH)-D5 at doses of 0.1–1 μM (Figure 2). However, at doses >5 μM, 1α(OH)D5 exhibited growth suppression of MCF-12F cells. This suggested that growth inhibition of normal breast epithelial cells requires higher 1α(OH)D5 dose than is necessary for the suppression of the DMBA-induced precancerous lesions (1 μM) in organ culture (9). Interestingly, there was a slight growth stimulatory (20–40% increase in cell number compared with control) effect during the first 3 days of treatment with 1α(OH)D5 and 1α,25$(OH)_2D_3$. These results suggest that 1α(OH)D5 and 1α,25$(OH)_2D_3$ do not inhibit growth of normal-like MCF-12F cells and mammary glands in culture.

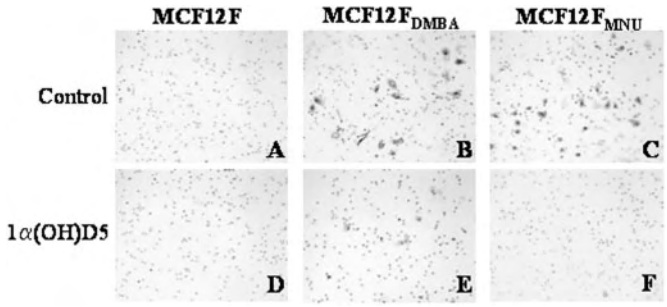

Fig. 3. Comparison of the effect of 1α(OH)D5 (1 μM) on the invasive potential of MCF-12F, MCF-12F$_{DMBA}$ and MCF-12F$_{MNU}$ cells. Matrigel® coated membrane in Boyden chambers were stained 48 h after 1α(OH)D5 treatment. (**A**) and (**D**), MCF-12F control and 1α(OH)D5; (**B**) and (**E**), MCF-12F$_{DMBA}$ control and 1α(OH)D5; (**C**) and (**F**), MCF-12F$_{MNU}$ control and 1α(OH)D5, respectively.

Table I. Cell cycle analysis of normal and transformed MCF-12F cells

Cell line	G-1 (%)	G-2 (%)	S (%)	G-1–G-2
MCF-12F				
Control	66.1 ± 2.1	24.6 ± 1.3	9.3 ± 1.2	2.7
1α(OH)D5[a]	65.1 ± 3.4	27.1 ± 2.0	7.8 ± 0.7	2.4
MCF-12F$_{DMBA}$				
Control	56.2 ± 2.8	6.3 ± 0.9	37.5 ± 2.2	8.9
1α(OH)D5[a]	70.3 ± 2.5*	11.2 ± 1.6	10.5 ± 1.4*	6.3
MCF-12F$_{MNU}$				
Control	57.1 ± 2.3	18.9 ± 1.1	24.0 ± 1.8	3.0
1α(OH)D5[a]	74.7 ± 1.9*	17.7 ± 2.3	11.6 ± 0.5*	4.2

[a]Cells treated with 1α(OH)D5 for 3 days.
*Significantly different from the control ($P < 0.05$); mean (± SEM) were compared using two-tailed *t*-tests.

Transformation of MCF-12F cells

It has been previously established that the carcinogen-transformed mouse mammary glands respond to growth inhibitory action of 1α(OH)D5 (9). To determine whether 1α(OH)D5 would similarly inhibit the growth of carcinogen-transformed human breast epithelial cells, MCF-12F cells were transformed using the carcinogens DMBA and MNU. The resulting cell lines MCF-12F$_{DMBA}$ and MCF-12F$_{MNU}$ exhibited a 3-fold faster growth rate than the parent cells as determined by the cell count. The transformed cells showed rounded and raised morphology as compared with the more polygonal and flattened appearance of the parent MCF-12F cells under a phase contrast microscope (data not shown). The transformed cells lacked contact inhibition and grew in multiple layers. Unlike the normal cells, the transformed cells did not require presence of epidermal growth factor in media to proliferate. In short, the carcinogen-transformed cells showed growth characteristics different from that of the normal-like MCF-12F cell line.

In order to determine whether the transformed cells were truly different from the normal MCF-12F cells, the invasion properties of these cells were compared using the Boyden chamber assay. As shown in Figure 3, the transformed (but not the parent) cells showed invasion through the Matrigel® coated membrane in the Boyden chambers. MCF-12F$_{DMBA}$ showed less invasive potential compared with the MCF-12F$_{MNU}$ cells. The percentage of cells invading through the membrane was ~14% for MCF-12F$_{MNU}$ and 12% for MCF-12F$_{DMBA}$ cells. Treatment with 1α(OH)D5 reduced (<2% cells invaded) the invasive potential of MCF-12F$_{DMBA}$ cells. On the other hand, it prevented the invasion of MCF-12F$_{MNU}$ cells through the membrane altogether (Figure 3). Although, these results indicate transformation of the MCF-12F cells by the carcinogens, the transformed cells failed to form tumors in athymic mice (data not shown). Thus, we conclude that the transformed cells are probably precancerous but have not acquired the ability to induce tumors in nude mice.

Effects of 1α(OH)D5 on the growth of normal and transformed MCF-12F cells

The normal-like MCF-12F cells did not show growth inhibition upon 1α(OH)D5 treatment at a dose of 1 μM. However, the growth of MCF-12F$_{DMBA}$ cells was inhibited significantly by 20% and MCF-12F$_{MNU}$ cells by 26% ($P < 0.05$) upon 1α(OH)D5 treatment for 3 days as compared with the controls. Similarly, 1α,25(OH)$_2$D$_3$ also inhibited the growth of transformed MCF-12F cells, while it had no growth inhibitory effect on the parent cell line at a dose of 0.01 μM. Since the cell count measures total cells adherent to the plate but does not take into account cells that are not viable, the growth inhibitory effects of 1α(OH)D5 were examined using the MTT assay (Figure 2). The growth of MCF-12F$_{DMBA}$ cells was inhibited by 40%, while MCF-12F$_{MNU}$ cells by 60% ($P < 0.05$) after 3 days of 1α(OH)D5 treatment. Both cell growth assays showed MCF-12F$_{MNU}$ cells to be more sensitive to treatment with 1α,25(OH)$_2$D$_3$ and 1α(OH)D5 than the MCF-12F$_{DMBA}$ cells. Moreover, in both assays there was no growth inhibition observed in the normal-like MCF-12F cells.

Cell cycle analysis revealed no significant difference in any of the stages of the cell cycle between control and 1α(OH)D5-treated MCF-12F cells (Table I). On the other hand, 1α(OH)D5-treated MCF-12F$_{DMBA}$ cells showed 25% increase in the G-1 phase of the cell cycle as compared with the controls after 3 days of treatment. In addition, the S phase was decreased by 70% in the treated cells. Similarly, MCF-12F$_{MNU}$ cells showed an increase of 30% in G-1 phase upon treatment with 1α(OH)D5 and a 50% decrease in the S phase compared with controls. Taken together, these results suggest growth inhibitory action of 1α(OH)D5 on transformed MCF-12F, but not on normal-like MCF-12F cells.

Since the transformed MCF-12F cells responded to 1α(OH)D5-mediated growth inhibition, differential gene expression analyses using Human Atlas 8K chip was obtained. Results showed that there were 369 genes differentially expressed between MCF-12F and MCF-12F$_{MNU}$ cells. Several cell cycle regulatory genes and genes related to extracellular matrix proteins were identified. Among the genes related to cell cycle regulation, TGFα, prohibitin and pituitary tumor transforming factor-1 were significantly upregulated; whereas, the Bcl2-like 1 gene and E2F-4 were downregulated. Treatment of MCF-12F$_{MNU}$ cells with 1α(OH)D5 (1 μM) resulted in the reversal of their expression (Table II). Prohibitin was selected as one of the potential target genes for further analysis. Not only does it interact with a variety of cell cycle regulatory proteins including E2F, pRb and p53 (20), prohibitin is a well conserved protein and the orthologs have been identified in plants, yeast and Drosophila. Results for prohibitin from microarray analyses were further confirmed by RT–PCR analysis. As shown in Figure 4, MCF-12F$_{MNU}$ shows

Table II. Differential gene expression analyses using Atlas 8K array*

Selected genes	Analysis 1 MCF-12F$_{MNU}$ versus MCF-12F	Analysis 2 MCF-12F$_{MNU}$ D5 versus MCF-12F$_{MNU}$
Prohibitin	4.1 up	2.4 down
Bcl2-like 1	2.8 down	2.5 up
TCTP-1	17.8 down	16.1 up
ODC Antizyme 1	2.1 down	2.1 up
TIMP-1	3.1 down	2.6 up
Glutathione Peroxidase	2.7 down	3.0 up
HSP27	2.7 up	4.0 down
Rho GDP Dissociation inhibitor α	6.5 down	5.9 up
Systatin (Stefan B)	3.4 down	2.9 up

*The microarray analyses were commercially performed by Clontech, (Palo Alto, CA). Normalization of the data was carried out by using global sum method with a coefficient of 1.35. Any differential expression of <2-fold was considered as insignificant.

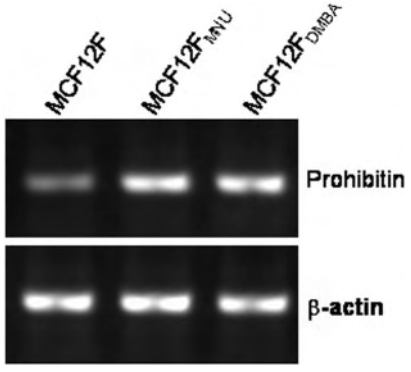

Fig. 4. RT–PCR analysis of steady state levels of prohibitin mRNA in normal and transformed MCF-12F cells. Prohibitin expression was 2-fold higher in the transformed cells as compared with the parent MCF-12F cells.

significant upregulation of prohibitin mRNA as compared with MCF-12F cells.

Effects of 1α(OH)D5 treatment on breast cancer cell growth

Since 1α(OH)D5 treatment elicited growth inhibition in transformed but not normal-like MCF-12F cells, commercially available breast cancer cell lines were used to further understand the growth inhibitory nature of 1α(OH)D5 action. Initially, cell growth was measured using the cell counter. It showed significant inhibition of BT-474 cell growth (50%, $P < 0.05$), while MCF-7 cells showed a modest inhibition (20%) after 72 h of 1α(OH)D5 treatment. This was further confirmed by the MTT assay. Results showed significant growth inhibition (50%, $P < 0.05$) in both estrogen-responsive BT-474 and MCF-7 cell lines after 3 days of treatment (Figure 5). On the other hand, the estrogen-independent cell lines MDA-MB-231 and MDA-MB-435 did not show any growth inhibitory effects of 1α(OH)D5 treatment.

Cell cycle analysis of the 1α(OH)D5-treated breast cancer cells showed results consistent with the cell growth studies (Table III). 1α(OH)D5-treated MDA-MB-231 and MDA-MB-435 cells did not show any significant change in the percentage of cells in different phases of cell cycle compared with the controls. Whereas, BT-474 cells showed 22% increase in cells in G-0/G-1 phase by Day 3 and 25% by Day 7 of treatment as compared with the control. Cells in the S phase, subsequently, was decreased by 71% on Day 3 and 62% on Day 7 of

Table III. Cell cycle analysis of breast cancer cell lines

Cell line	G-1 (%)	G-2 (%)	S (%)	G-1–G-2
BT-474				
Control	65.3 ± 2.3	18.6 ± 1.1	16.1 ± 2.8	3.5
1α(OH)D5[a]	79.4 ± 2.1*	5.3 ± 0.6*	15.3 ± 2.3	15.0
MCF-7				
Control	66.8 ± 2.3	11.4 ± 1.2	21.5 ± 0.8	5.9
1α(OH)D5[a]	78.1 ± 2.5*	8.4 ± 1.3	12.1 ± 0.3*	9.3
MDA-MB-435				
Control	22.8 ± 1.4	45.9 ± 4.3	31.3 ± 1.8	0.5
1α(OH)D5[a]	21.1 ± 1.9	55.3 ± 3.6	23.6 ± 3.7	0.4
MDA-MB-231				
Control	66.2 ± 3.1	10.1 ± 0.4	23.7 ± 3.2	6.6
1α(OH)D5[a]	65.5 ± 2.9	9.7 ± 0.9	24.9 ± 0.9	6.8

[a]Cell lines treated with 1α(OH)D5 for 3 days.
*Significantly different from the control ($P < 0.05$); mean (±SEM) were compared using two-tailed *t*-tests.

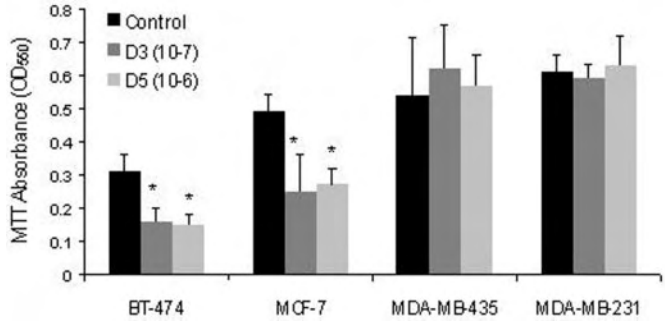

Fig. 5. Effect of 1α(OH)D5 on the viability of various breast cancer cell lines after 3 days of treatment as determined by the MTT assay. Cells were incubated with 1 μM 1α(OH)D5 for 72 h in a 96-well plate; 16 wells per group were used. *P-value <0.05. Mean (±SEM) were compared using two-tailed *t*-tests.

treatment as compared with their respective controls. Similarly, MCF-7 cells showed 22% increase in the percentage of cells in G-0/G-1 phase of the cell cycle and 45% decease in the S phase by Day 7, suggesting a gradual accumulation of the cells in G-1/G-0 phase. Thus, the treatment with 1α(OH)D5 resulted in an inhibitory action on the proliferation of hormone-responsive breast cancer cells, while no effect was observed on hormone-resistant breast cancer cells.

Effects of 1α(OH)D5 treatment on expression of cell cycle-related proteins in breast cancer BT-474 cells

Studies with 1α(OH)D5 treatment of normal-like and breast cancer cells indicated a growth inhibitory action of 1α(OH)D5 on hormone-sensitive breast cancer cells as well as early stage transformed breast epithelial cells. Cell cycle analysis of responsive cell lines showed increased proportion of 1α(OH)D5-treated cells in the G-0/G-1 phase of the cell cycle indicating a possible effect of 1α(OH)D5 on genes that regulate the transition of cells from G-1 to S phase. Cyclins regulate the activity of their dependent kinases (CDK), while the inhibitors (CDKI), such as p21^{Waf-1} and p27^{Kip-1}, inhibit the activity of CDKs. In order to determine the effect of 1α(OH)D5 on the expression of these cell cycle regulatory proteins, RT–PCR and western blot analysis were conducted on control and treated BT-474 cells. BT-474 cells were chosen for expression studies owing to their established cancer status and their responsiveness to 1α(OH)D5 treatment.

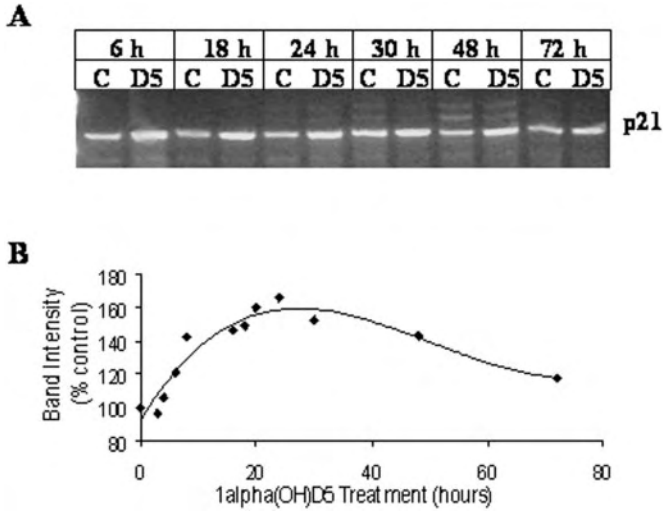

Fig. 6. Effect of 1α(OH)D5 treatment on transcription of CDK inhibitor $p21^{Waf-1}$ in BT-474 cells. BT-474 cells were treated with 1 μM 1α(OH)D5 or control for various time points. Total RNA was extracted and subjected to RT–PCR. (**A**), RT–PCR showing levels of $p21^{Waf-1}$ mRNA from control and 1α(OH)D5-treated (1 μM) cells for each time point, respectively. (**B**), Change in levels of $p21^{Waf-1}$ transcript with 1α(OH)D5 treatment, adjusted for control and β-actin. The results are expressed as percent expression relative to appropriate controls. The graph was generated using polynomial regression.

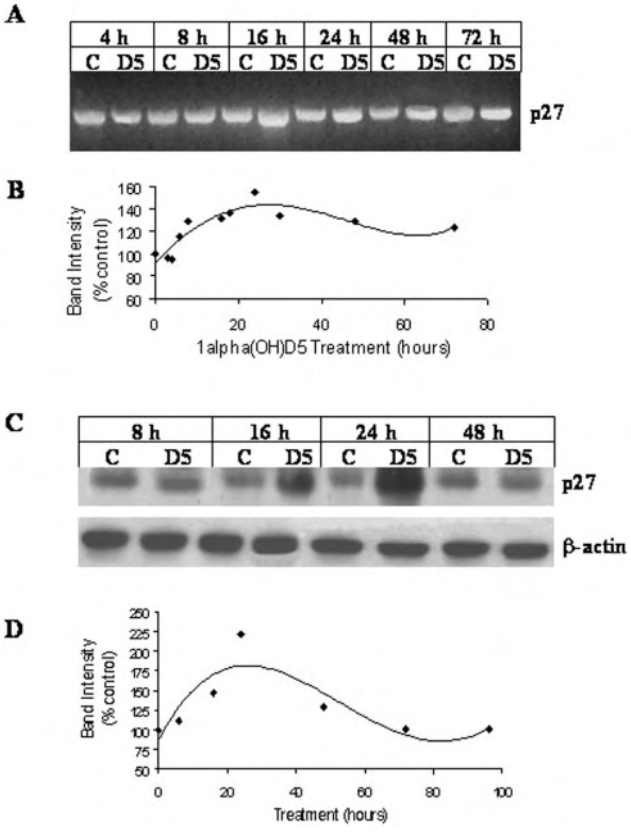

Fig. 7. Effect of 1α(OH)D5 treatment on the levels of CDK inhibitor $p27^{Kip-1}$ in BT-474 cells. (**A**) and (**C**), RT–PCR and western blots showing levels of $p27^{Kip-1}$ from control and 1α(OH)D5 -treated cells (1 μM) for each time point, respectively. (**B**) and (**D**), Change in levels of $p27^{Kip-1}$ transcription and expression, respectively, adjusted for control and β-actin. The results are expressed as percent expression relative to appropriate controls. The graph was generated using polynomial regression.

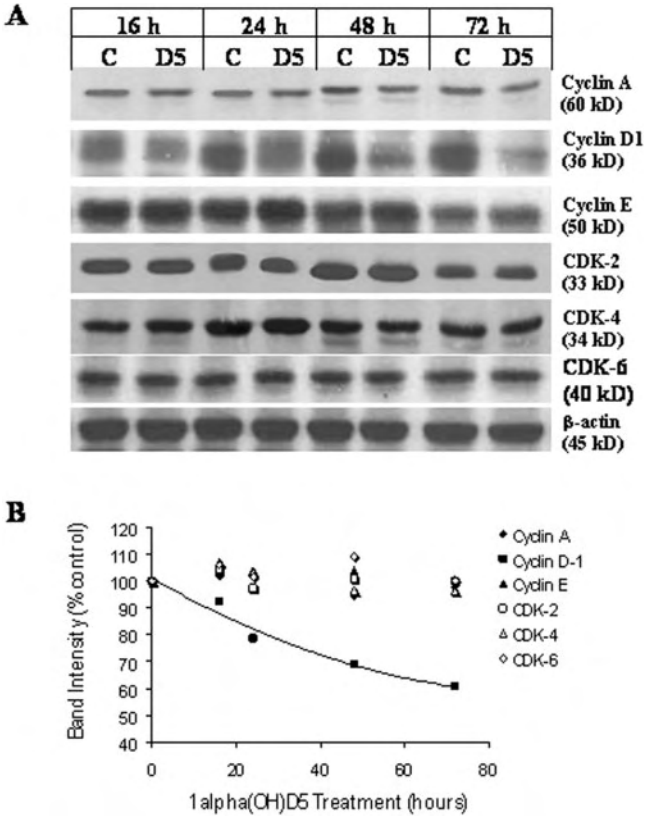

Fig. 8. Western blots showing expression of cell cycle regulatory cyclins and CDKs in breast cancer BT-474 cells in response to 1 μM 1α(OH)D5 treatment (**A**). The results are expressed as percent control after adjusting for β-actin. The graph represents the band density data in an *XY* scatter plot (**B**).

The major checkpoints at G-1 to S transition in the cell cycle are CDKI, $p21^{Waf-1}$ and $p27^{Kip-1}$. In order to determine whether 1α(OH)D5 treatment can result in modulation of the $p21^{Waf-1}$ transcription, control and 1α(OH)D5-treated BT-474 cells were sampled at intervals and total RNA was extracted and subjected to gene-specific RT–PCR. The results showed that in 24 h, $p21^{Waf-1}$ message was increased by 70% of the control (Figure 6A and B). Transcript levels of another CDKI $p27^{Kip-1}$, were found to be increased by 60% in the 24 h following 1α(OH)D5 treatment in BT-474 cells (Figure 7A and B). This increase in $p27^{Kip-1}$ message was followed by a 2-fold increase in the expression of $p27^{Kip-1}$ protein as determined by the western blot analysis (Figure 7C and D). At the mRNA level, $p21^{Waf-1}$ levels were upregulated at the beginning of 8 h of 1α(OH)D5 treatment and reached a peak at 24 h. While the expression of $p27^{Kip-1}$ peaked at 24 h following 1α(OH)D5 treatment, the initial increases were observed at 16 h of treatment.

Although the CDKIs $p21^{Waf-1}$ and $p27^{Kip-1}$ inhibit the activity of the CDKs, the cyclins enhance the activity of these kinases. The major CDKs and their corresponding cyclins for G-1 to S transition of cell cycle include cyclins A, D1 and E, with CDKs 2, 4 and 6. Expression of these cyclins and their dependent kinases was determined in BT-474 cells following 1α(OH)D5 treatment at different intervals using western blots. No significant changes were found in the expression of these G-1 associated cyclins and CDKs, with the exception of cyclin D1. Cyclin D1 expression started to decrease by 20% at 24 h of 1α(OH)D5 treatment, and by Day 3 it was reduced to 60%

of the control (Figure 8). Although 1α(OH)D5 treatment of BT-474 cells did not down regulate CDKs, it appears that their activity was modulated by 1α(OH)D5 treatment via upregulation of $p21^{Waf-1}$ and $p27^{Kip-1}$.

Discussion

Previous studies have shown growth inhibitory effects of 1α(OH)D5 treatment both *in vivo* and *in vitro* (9,13). The *in vivo* data also indicated that 1α(OH)D5 was especially effective in reducing tumor growth when present during the promotion stage of the carcinogenesis (10). However, the effects of 1α(OH)D5 on the growth and morphology of normal mammary glands and cells were not known. Toxicity studies in animal models had shown low calcemic activity and relative safety of 1α(OH)D5 (13). To further understand whether 1α(OH)D5 would inhibit growth or alter morphology of normal breast epithelial cells, we treated cultured normal mouse mammary glands and normal-like human breast epithelial cells MCF-12F with 1α(OH)D5 at a dose that was found to be effective in preventing preneoplastic transformation of mouse mammary glands. The 1α(OH)D5-treated normal mouse mammary glands and MCF-12F cells had growth and morphology similar to the controls. 1α(OH)D5, at doses effective in suppressing cancer cell growth *in vitro*, did not suppress the growth of normal tissue or normal-like breast epithelial cells. Interestingly, at higher doses ($\geq$5 μM) 1α(OH)D5 showed growth inhibitory effect in MCF-12F cells (data not shown). This suggests that a dose higher than the dose effective in human breast cancer cells would be required to inhibit growth of normal epithelial cells. This is consistent with data from other studies (8), where 1α,25$(OH)_2D_3$ and its analogs, EB-1089 and CB-1093, in nanomolar concentration did not effect growth of VDR negative normal breast epithelial cells derived from VDR knockout (VDRKO) mouse. However, at micromolar concentration, signs of early apoptosis and growth arrest were observed. Some studies have also shown a slight growth stimulatory effect of 1α,25$(OH)_2D_3$ and EB-1089 in human breast epithelial cells (21). We observed similar growth stimulatory effect of 1α(OH)D5 in MCF-12F cells in the first 3 days of treatment. However, this stimulatory growth effect on MCF-12F cells was diminished by Day 7 of treatment. In another study, lung cancer cells xenografted in VDRKO and wild-type mice (22) showed that lung cancer cell metastasis was reduced in VDRKO mice. VDRKO mice accumulate 1α,25$(OH)_2D_3$ due to lack of VDR-mediated negative feedback regulation of 24-hydroxylase. The reduction in metastasis of lung cancer cell xenograft in VDRKO mice was attributed to the high levels of 1α,25$(OH)_2D_3$, which possibly affected pathways independent of VDR.

Transformation of breast cells occurs through a series of events leading to deregulation of signal transduction pathways, abnormal growth signals and aberrant expression of genes that ultimately transform the cells into invasive type. Use of chemical carcinogen is an effective method to transform normal cells in order to study and compare the effects of a potential chemopreventive agent on the growth of semi-transformed or transformed cells. In the present study, the carcinogen-induced transformation of MCF-12F using DMBA and MNU was performed with the same objective. As reported in the results, both $MCF\text{-}12F_{DMBA}$ and $MCF\text{-}12F_{MNU}$ cells showed growth characteristics different from the parent cell line. In addition to these differences, the transformed cells also showed growth inhibition with 1α(OH)D5 treatment at a dose of 1 μM. There was no growth inhibitory effect of 1α(OH)D5 on MCF-12F cell growth at that dose. These results are consistent with earlier reports indicating that 1α,25$(OH)_2D_3$ and EB-1089 in nanomolar concentrations blocked mitogenic effects of fibroblast-conditioned medium on MCF-7 tumor cells but not on normal breast epithelial cells (23). One of the basic requirements for a chemopreventive agent is that it should not elicit adverse growth effects on normal tissues of the body at chemopreventive doses. Our results indicate that 1α(OH)D5 is a likely candidate in this respect. The significance of prohibitin in breast epithelial or cancer cells is not clear. It appears that prohibitin binds to VDR and enhances the responsiveness to vitamin D in VDR-positive breast cancer cells (unpublished).

Although 1α(OH)D5 induced growth inhibition in certain breast cancer cells, it did not affect all types of breast cancer cells. At a dose of 1 μM, 1α(OH)D5 showed significant growth inhibition of hormone-responsive BT-474 and MCF-7 cells. On the other hand, the estrogen-resistant cell lines MDA-MB-231 and MDA-MB-435 did not show any growth inhibitory effects of 1α(OH)D5 treatment. Hansen and colleagues (24) had shown that despite its inability to inhibit growth of MDA-MB-231 cells, 1α,25$(OH)_2D_3$ reduced the invasive potential of MDA-MB-231 by 50% as determined by the Boyden Chamber assay. However, 1α(OH)D5 did not alter the invasive potential of MDA-MB-231 cells through the Matrigel® coated membranes in Boyden chambers (unpublished data). Thus, the treatment with the optimal chemopreventive dose of 1α(OH)D5 resulted in an inhibitory action on proliferation of hormone-responsive breast cancer cells, while no effect was observed on hormone-resistant breast cancer cells. One explanation of the inability of 1α(OH)D5 to inhibit growth of hormone-resistant cell lines could be the levels of VDR present. BT-474 cells express high levels of VDR and MCF-7 express moderate levels, while MDA-MB-231 express low levels (25). VDR appears to be essential for the antiproliferative response of vitamin D_3 and its analogs in the mammary tissue. This is consistent with the previous study where estrogen receptor (ER)-negative but VDR-positive UISO-BCA-4 breast cancer cells responded to growth inhibition by 1α(OH)D5 (13). The understanding of the nature of growth inhibitory response of 1α(OH)D5 would serve to identify suitability for its clinical use in certain types and stages of breast cancer.

The growth inhibitory action of 1α(OH)D5 in the transformed and cancer breast cells was accompanied by accumulation of cells in G-0/G-1 phase of the cell cycle. The increase in the percentage of cells in G-1 phase is indicative of cell cycle arrest that might lead to either differentiation or cell death. Several researchers have shown that 1α,25$(OH)_2D_3$ and some of its analogs induce G-1 arrest in responsive breast cancer cells (26–28). The 1α(OH)D5-mediated growth arrest is preceded by modulation of several checkpoints in the cell cycle. Therefore, various cell cycle regulatory factors that are involved in G-1/S phase transition were studied to determine the effects of 1α(OH)D5 on these factors. In BT-474 breast cancer cells, the expression of $p21^{Waf-1}$ and $p27^{Kip-1}$ was upregulated within 24 h following 1α(OH)D5 treatment. These two CDKIs inhibit cell cycle progression from G-0–G-1 to S phase by downregulating cyclins A, D and E, which in turn, leads to reduced CDK-2, -4 and -6 activity. In cross-sectional studies, loss of $p21^{Waf-1}$ and $p27^{Kip-1}$ in breast

and other cancer tissue samples has been reported (27) and expression of these two CDKIs has been used as a biomarker for cancer progression. Consistent with our data, other reports have also shown that 1α,25(OH)$_2$D$_3$ and EB-1089 upregulated p21^{Waf-1} expression in several breast cancer cell lines including MCF-7, T-47D, ZR-75-1 and BT-20 (26,29). One of the main targets for action of p21^{Waf-1} and p27^{Kip-1} is the reduction of cyclin D1 expression and/or activity. Our results have similarly indicated that cyclin D1 expression started to decrease within 24 h of 1α(OH)D5 treatment in BT-474 cells. Other G-1/S phase cyclins and their respective CDK levels were not affected by 1α(OH)D5 treatment. Reports by several researchers, however, suggest that the activities of CDKs 2 and 4, regardless of their levels, were significantly decreased upon treatment with 1α,25(OH)$_2$D$_3$ and EB-1089 in MCF-7, T-47D, ZR-75-1 and BT-20 breast cancer cells along with a decrease in cyclins A and D1 levels (26,28). Treatment of BT-474 cells with 1α(OH)D5 showed similar effects on expression of various cell cycle-related genes as reported of vitamin D$_3$ and some of its analogs. This modulation of gene expression by 1α(OH)D5 was followed by growth arrest of BT-474 cells. A recent report showed that in laryngeal carcinoma cells the antiproliferative activity of 1α,25(OH)$_2$D$_3$ was due specifically to induction of CDKI p21^{Waf-1}, although both p21^{Waf-1} and p27^{Kip-1} were upregulated by 1α,25(OH)$_2$D$_3$ treatment (30).

In conclusion, the growth inhibitory effect of 1α(OH)D5 was achieved in breast cancer cells at a dose that was found to be ineffective in normal-like breast epithelial cells. This selective action could be a result of potential antiestrogenic action of 1α(OH)D5 combined with altered expression of various potential target genes (31). Taken together, these data indicate that, in human breast epithelial tissues, 1α(OH)D5 is selective for its effects on precancerous or cancer cells, but shows no effect on normal breast epithelial cell growth. Moreover, the growth inhibitory effect is elicited by upregulation of cell cycle checkpoints that arrest the cells in G-0/G-1 phase, thereby reducing cancer cell proliferation. Such selective growth inhibition of cancer cells is a useful property for a successful chemopreventive agent in clinical settings.

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Conflict of Interest Statement: None declared.

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Research Article

Prohibitin Is a Novel Target Gene of Vitamin D Involved in Its Antiproliferative Action in Breast Cancer Cells

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Abstract

Previously, we showed that *N*-methyl-*N*-nitrosourea–transformed MCF12F breast epithelial cells exhibited differential expression of several genes, including up-regulation of prohibitin and elevated sensitivity to a relatively noncalcemic vitamin D analogue, 1α-hydroxyvitamin D5 [1α(OH)D_5]. In this report, we evaluated the functional significance of prohibitin in relation to the cellular response to vitamin D. The *in silico* screening for putative transcription factor binding sites identified two vitamin D receptor (VDR)/retinoid X receptor binding sites in the 1-kb promoter region of prohibitin. Prohibitin up-regulation by 1α(OH)D_5 treatment at both transcriptional and translational levels was confirmed by real-time reverse transcription-PCR and Western blot analysis in breast cancer cells, identifying prohibitin as a vitamin D target gene. Confocal microscopic analysis showed that prohibitin was localized in the nuclei of MCF-7 cells and a portion of prohibitin was colocalized with VDR, but direct physical interaction between VDR and prohibitin in cell lysates was not detectable. In MCF-7 cells expressing tetracycline-inducible prohibitin (*Tet-On* model), the overexpression of prohibitin inhibited cell proliferation and enhanced vitamin D–induced antiproliferative activity. Knockdown of prohibitin was accompanied by increased number of cells incorporating bromodeoxyuridine in the whole population and increased cell distribution in the S phase of cell cycle. In addition, prohibitin level had no significant effect on the vitamin D–induced transactivation of *CYP24*, a VDR target gene. This is the first report to suggest that prohibitin serves as a novel vitamin D target gene, which is involved in the antiproliferative action of vitamin D without affecting CYP24 transactivation in breast cancer cells. (Cancer Res 2006; 66(14): 7361-9)

Introduction

It is now well recognized that the active metabolite of vitamin D plays a significant role as a modulator of cell proliferation and differentiation in addition to its principal function in bone mineralization. However, its clinical use in cancer prevention and therapy is hampered due to its toxic effects at efficacious concentration. Therefore, considerable effort has been diverted toward designing and synthesizing chemical analogues of vitamin D that are less calcemic and more potent in inhibiting growth of cancer cells. We synthesized one such less calcemic analogue, 1α-Hydroxy-24-ethyl-cholecalciferol [1α(OH)D_5], which has shown promising chemopreventive effect in mouse mammary organ culture system (1, 2), chemically induced rat mammary carcinogenesis and breast cancer xenograft model using athymic mice (3, 4). To this end, 1α(OH)D_5 is being developed for phase I clinical trials for breast cancer patients. Our previous effort to identify transformation-associated genes using microarray analysis found that several genes were up-regulated in carcinogen-transformed human breast epithelial cells compared with the untransformed cells (MCF12F), including prohibitin, TCTP1, and thioredoxin (3, 5). Because it is well known that vitamin D arrests epithelial cells in G_1 phase of the cell cycle whereas prohibitin has been reported to have tumor-suppressive and antiproliferative effects, we selected prohibitin to determine if it has any functional significance in vitamin D action. The prohibitin gene encodes a protein of 275 amino acids with a molecular mass of 28 to 30 kDa. It is highly evolutionarily conserved and mapped to chromosome 17q12-21 (6). Its protein is localized into the inner membrane of the mitochondria, where it might have a role as a mitochondrial chaperon protein in a complex with BAP37 in the maintenance of mitochondrial function and protection against senescence (7–9). In addition, prohibitin also plays a regulatory role within the cell cycle, although the precise role of the protein in cell cycle regulation is not well understood (10). Earlier work considered prohibitin as a potential tumor suppressor gene because microinjection of prohibitin transcripts resulted in growth arrest in HeLa cells (11). Prohibitin has also been reported to be localized to the nuclei of breast cancer cells (12) and to mediate hormone response in prostate cancer and ovary granulosa cells (13, 14). There is evidence for the interaction of prohibitin with the cell cycle checkpoint molecules, including E2F (15), p53 (12), and pRB (16, 17), and the overexpression of prohibitin can modulate transcription of multiple genes in the transfection experiments. This suggests a potential mechanism of prohibitin function in cell cycle regulation (12, 15, 17). Recent reports indicate that prohibitin is up-regulated in tumor cells compared with normal cells (18–20). Moreover, it has been reported as one of the target genes for c-Myc (21, 22). The consistently higher levels of prohibitin in cancer cells may be due to the transactivation of *c-Myc* gene (8). Thus, it seems that prohibitin is involved in diverse cellular processes, including proliferation, stress (7), cell migration (23), etc., associated with its different localization in the cells; however, its nuclear function is still far from clear. Our attempt to characterize the role of prohibitin in vitamin D receptor (VDR) function led to the identification of prohibitin as a novel vitamin D target gene that is involved in the antiproliferative action of vitamin D without affecting CYP24 transactivation in breast cancer cells.

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Materials and Methods

Vitamin D analogue. $1\alpha(OH)D_5$ was synthesized according to the procedure described previously (1). $1\alpha(OH)D_5$ was dissolved in ethanol, and the stock solution of 10 mmol/L $1\alpha(OH)D5$ in ethanol was stored in −80°C freezer. The appropriate controls for each experiment consisted of treatment with the vehicle (ethanol) used at a concentration <0.01% of total cell culture medium. $1,25(OH)_2D_3$ was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Cell culture. MCF-7, BT474, and MDA-MB231 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured as described previously (24). MCF-7 cells expressing tetracycline-inducible prohibitin (tagged by c-Myc) were grown in DMEM supplementd with 10% tetracycline-free fetal bovine serum (FBS; Clontech, Palo Alto, CA), 25 μg/mL Zeocin (Invitrogen, Carlsbad, CA), and 0.25 μg/mL Blasticidine (Invitrogen) as described (12). Prohibitin was induced by incubation of cells with 1 μg/mL tetracycline or 20 ng/mL doxycycline in the MCF-7 *Tet-On* model. A day before treatment, cells were seeded and incubated for 24 hours in growth medium to allow cells to attach and the serum in the medium was reduced to 5%. Then, cells were then treated with 0.5 μmol/L $1\alpha(OH)D_5$ or 10 nmol/L $1,25(OH)_2D_3$ for different times. For long treatments, medium was changed every other day.

RNA extraction and real-time reverse transcription-PCR. Total RNA extraction and reverse transcription reaction were done as described previously (24). RNA was further subjected to DNase I (Ambion, Austin, TX) digestion and purification using RNeasy Mini kit (Qiagen, Valencia, CA) before reverse transcription. Real-time PCR was done with 1 μL reverse transcription product in a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA) by using iQ SYBR Green PCR Supermix (Bio-Rad) according to the manufacturer's guidelines. The PCR cycling conditions used were as follows: 40 cycles of 15 seconds at 95°C, 15 seconds at 60°C, and 20 seconds at 72°C. Fold inductions were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is $\Delta Ct_{(treatment)} - \Delta Ct_{(control)}$, ΔCt is $Ct_{(target\ gene)} - Ct_{(actin)}$, and Ct is the cycle at which the threshold is crossed. The gene-specific primer pairs (and product size) for the gene analyzed here were as follows: *prohibitin*, 5′-ACCACGTAATGTGCCAGTCA-3′ (forward) and 5′-TAGTCCTCTCC-GATGCTGGT-3′ (reverse; 126 bp); *CYP24*, 5′-CTCAGCAGCCTAGTGCA-GATT-3′ (forward) and 5′-ACTGTTTGCTGTCGTTTCCAC-3′ (reverse; 122 bp); and *β-actin*, 5′-CTCTTCCAGCCTTCC-TTCCT-3′ (forward) and 5′-AGCACTGTGTTGGCGTACAG-3′ (reverse; 116 bp). PCR product quality was monitored using post-PCR melt curve analysis.

Cell proliferation assay. Cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, direct cell counting, and bromodexyuridine (BrdUrd) incorporation assay. MTT assay was described previously (24). Cells (1,000) per well were initially seeded in 96-well plates for MTT assay. For direct cell counting, cells were initially seeded in six-well (5,000 per well) or 12-well (2,000 per well) plates; after treatment, cells were trypsinized and cell number was determined by direct counting using Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA) with size setting at 8 to 12 μm. For BrdUrd incorporation assay, 10 μmol/L BrdUrd (Calbiochem, La Jolla, CA) was added to the medium 2 hours before harvesting cells. Cells were trypsinized, washed with PBS, and fixed in 1% paraformaldehyde in PBS for 15 minutes followed by incubation in PBS containing 0.2% Tween 20 for 30 minutes at 37°C. Cells were then incubated with mouse monoclonal anti-BrdUrd antibody (Calbiochem) overnight at 4°C, washed twice, and incubated with FITC-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After three washes with PBS, cells were subjected to fluorescence-activated cell sorting (FACS) analysis; a total of 20,000 events were measured per sample. BrdUrd incorporation assay is a more sensitive assay for cell proliferation, which was used to evaluate cell proliferation potential in MCF-7 cells transiently transfected with small interfering RNA (siRNA).

Fluorescent immunostaining and confocal microscopy. MCF-7 cells were grown on coverslips for 24 hours. Cells were washed in PBS and fixed in buffered formalin. Fixed cells were then washed thrice with PBS containing 0.1% Tween 20 (PBST), permeabilized in 0.2% Triton X-100/PBS for 5 minutes, blocked with 1% bovine serum albumin in PBST for 30 minutes, and then incubated with anti-VDR rat monoclonal antibody (mAb; 1:200; NeoMarkers, Fremont, CA) for 1 hour at room temperature. Cells were washed and incubated with FITC-labeled anti-rat secondary antibody for 1 hour. Antibody incubation steps were repeated with anti-prohibitin mouse mAb (NeoMarkers) and Texas red–labeled anti-mouse secondary antibody. After staining nuclei with 4′,6-diamidino-2-phenylindole (DAPI), cells were visualized with Zeiss LSM 510 (Zeiss, Thornwood, NY) confocal microscope and areas of colocalization were determined using LSM 510 software (Zeiss).

Coimmunoprecipitation and Western blot analysis. When cells grew to 50% to 70% confluence, cell lysates were prepared and subjected to either Western blot analysis as described previously (24) or coimmunoprecipitation. Mouse anti-prohibitin mAb was purchased from NeoMarkers. Rabbit polyclonal VDR antibody, c-Myc mouse mAb, and all secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For coimmunoprecipitation, cell lysates (500 μg) were incubated with VDR polyclonal antibody or c-Myc mAb in 300 μL binding buffer [50 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 100 μmol/L NaF, 200 μmol/L Na_3VO_4, proteinase inhibitor cocktail (Sigma-Aldrich), 1 mmol/L phenylmethylsulfonyl fluoride, 0.5% NP40] on a rocker platform for 3 hours at 4°C. Then, 30 μL protein A/G plus agarose (Santa Cruz Biotechnology) was added to each sample for overnight incubation. The agarose beads were washed five times with 600 μL of the same binding buffer, boiled in 20 μL SDS sample buffer, and subjected to Western blot analysis (24).

Expression plasmid and transfection. The prohibitin expression vector pcDNA3.1PHB was generated by PCR cloning using pcDNA3.1/V5-His TOPO TA Expression kit (Invitrogen). The open reading frame of prohibitin was isolated by PCR of full-length cDNA from MCF10A breast epithelial cells using primers containing start and stop codons [prohibitin, 5′-GGAAACATGGC-TGCCAAAGTG-3′ (start) and 5′-GCCCTCACTGGGGC-AGCTG-3′ (stop)]. Orientation and sequence of the construct were verified by direct sequencing. Transient transfection was done in the same culture medium containing 2% FBS using LipofectAMINE 2000 (Invitrogen) in 12-well plates per manufacturer's manual (24). Cells (1.5×10^5 per well) were transfected with 0.5 μg/well pcDNA3.1 empty vector (control) or 0.53 μg/well pcDNA3.1PHB expression vector. After 5 hours of incubation, the medium was replaced with medium containing 10 nmol/L $1,25(OH)_2D_3$ or ethanol (solvent control, 1 μL/10 mL medium), and cells were incubated for an additional 24 hours before analysis.

RNA interference. siRNA against prohibitin [prohibitin siRNA, 5′-CCCAGAAAUCACUGUGAAAdTdT-3′ (sense) and 5′-UUUCACAGUGAUUU-CUGGG-dTdT-3′ (antisense)] was designed to target the 3′-untranslated region at 974 to 993 bp of sequence NM_002634 and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Negative control siRNA [nonsilencing RNA, 5′-UUCUCCGAACGUGUCA-CGUdTdT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAAdTdT-3′ (antisense); Qiagen] has no homology to known mammalian genes. To knock down prohibitin by RNA interference (RNAi), 10^5 cells per well were seeded in six-well plates in culture medium containing 5% FBS and incubated overnight. Prohibitin siRNA and nonsilencing siRNA were transfected using the RNAi Carrier kit (Epoch Biolabs, Sugar Land, TX). Cells were first incubated with siRNA complex (siRNA at 80 nmol/L; siRNA to RNAi Carrier, 1:6) for 6 hours in Opti-MEM (Invitrogen) containing 2% FBS, and then FBS was increased to 5%. After overnight incubation, cells were incubated in fresh culture medium containing 5% FBS for 2 days, and then cells were subjected to BrdUrd incorporation assay or cell cycle analysis (25). Prohibitin knockdown was verified at the same time using reverse transcription-PCR (RT-PCR) and Western blot analysis.

Results

Prohibitin is a vitamin D target gene. Our earlier studies showed that prohibitin could be involved in cellular response to $1\alpha(OH)D_5$ in breast epithelial cells (5); therefore, a question was raised: is prohibitin a vitamin D target gene? To answer this

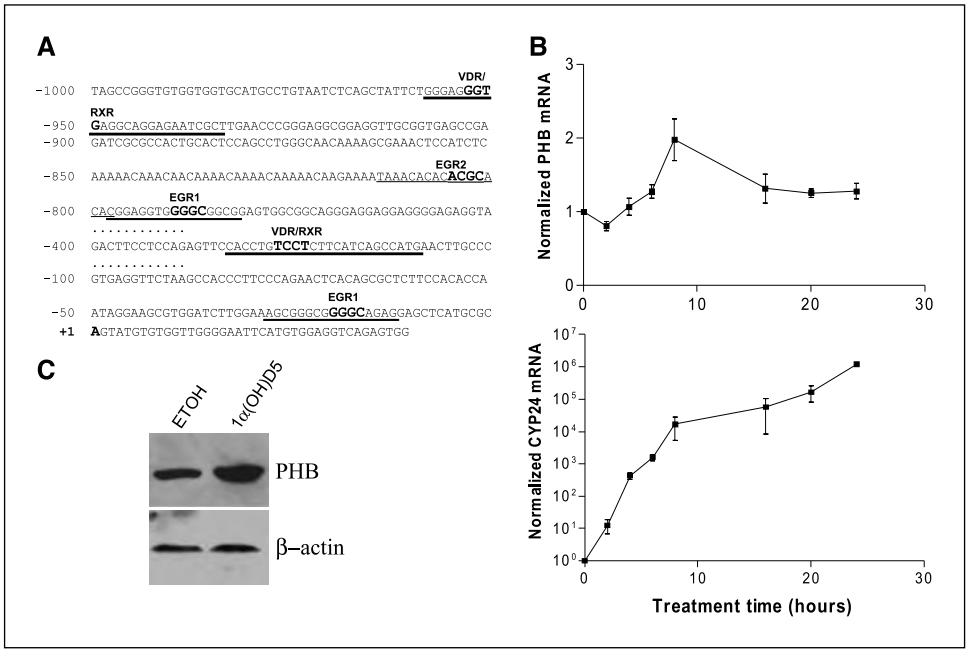

Figure 1. Prohibitin as a target gene of vitamin D. *A,* potential VDR/RXR binding sites in the promoter region of prohibitin gene. *Underlined,* core sequence (*bold font*) of the putative transcription binding sites for VDR/RXR and *EGR* (an identified VDR target gene) with high identity to the authentic core and matrix sequences as identified by MatInspector version 2.2 and Promo. Nucleotides are numbered negatively to the left of the sequence with nucleotide +1 corresponding to the transcription start site. *B,* effects of $1\alpha(OH)D_5$ on the expression of prohibitin (*PHB*; *top*) and CYP24 (*bottom*) mRNA in BT474 breast cancer epithelial cells. BT474 cells were treated for 0, 2, 4, 8, 16, 20, and 24 hours with 0.5 μmol/L $1\alpha(OH)D_5$. At each time point, RNA was extracted and analyzed by real-time RT-PCR. CYP24 mRNA expression was analyzed at the same time and served as a positive control for vitamin D response. The value at each time point represents the relative level of specific mRNA normalized to β-actin (the level of specific mRNA at time point 0 was set as 1). *Points,* mean of three independent experiments with duplicate analyses of each cDNA samples; *bars,* SE. *C,* effects of $1\alpha(OH)D_5$ (0.5 μmol/L, 24 hours) on prohibitin expression in BT474 cells. Total proteins (40 μg) extracted from cells are loaded for Western blot analysis. $1\alpha(OH)D_5$ treatment significantly increased prohibitin expression. β-Actin was used as an internal control.

question, we first analyzed the promoter region of prohibitin to examine if potential VDR binding sites are present. As shown in Fig. 1*A*, the 1-kb 5′-flanking sequence (−1,000/+40) of prohibitin was analyzed with several transcription binding site identification programs, including MatInspector (26), Promo (27), TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), etc. Both MatInspector (using Matrix Family Library version 6.0 with optimized similarity score set to be >0.75 for all vertebrates library) and Promo identified two potential DNA binding sites for VDR/retinoid X receptor (RXR) located at −958/−934 (core similarity, 0.785; matrix similarity, 0.796; positive strand) and −383/−359 (core similarity, 0.937; matrix similarity, 0.794; negative strand) of the 1-kb promoter sequence (Fig. 1*A*). In addition to the known binding sites, such as androgen receptor (13) and c-Myc (7), in the promoter region of prohibitin, MatInspector also identified multiple DNA binding sites for early growth response (EGR) in this region (Fig. 1*A*), which was previously identified as a vitamin D target gene in cultured hepatic cells (28, 29) and in breast cancer cells in our laboratory.[5] These analyses showed that prohibitin is a potential vitamin D target gene. To experimentally test if prohibitin is regulated by vitamin D, we selected BT474 cells to verify the results of *in silico* analysis. BT474 is estrogen receptor (ER)-positive and VDR-positive breast cancer cell line and is also very sensitive to $1\alpha(OH)D_5$ treatment (5). A time course analysis of prohibitin expression at transcriptional level showed that prohibitin mRNA level started going up at 4 hours after treatment with $1\alpha(OH)D_5$ (0.5 μmol/L) and peaked at 8 hours (~2-fold increase compared with basal level); at 24 hours after treatment, prohibitin level showed 20% to 30% increase compared with the basal level before treatment (Fig. 1*B, top*). *CYP24*, a direct vitamin D target gene, was analyzed at the same time to serve as a positive control for cellular response to vitamin D (Fig. 1*B, bottom*). Differing from prohibitin transcription, CYP24 showed continuous transactivation during the 24-hour time course of $1\alpha(OH)D_5$ treatment. To confirm if prohibitin protein is regulated by $1\alpha(OH)D_5$, Western blot analysis of prohibitin protein expression in BT474 cells after 24-hour $1\alpha(OH)D_5$ treatment was done (Fig. 1*C*). Prohibitin protein was significantly up-regulated by $1\alpha(OH)D_5$. These data clearly identify prohibitin as a vitamin D target gene.

Basal transcriptional level of prohibitin correlates to cellular sensitivity to vitamin D in breast cancer cells. Because prohibitin is a vitamin D target gene and was reported to have antiproliferative effect, we next asked if basal level of prohibitin is associated with any cellular sensitivity to vitamin D. CYP24 transactivation and antiproliferative effect by vitamin D were used as two variables for evaluation. Three breast cancer cell lines (ER-negative MDA-MB231, ER-positive BT474, and MCF-7 cells) with different sensitivities were selected. As shown in Fig. 2*A*, MDA-MB231 has the lowest basal prohibitin expression level, whereas BT474 has the highest basal prohibitin expression level, 6-fold higher compared with that of MDA-MB231 cells; the basal prohibitin level in MCF-7 is 2-fold higher than that in MDA-MB231 cells. The basal prohibitin level correlates to cellular sensitivity in response to vitamin D in these cell lines (Fig. 2*B* and *C*): $1\alpha(OH)D_5$ at 0.5 μmol/L dramatically induced CYP24 expression after 24-hour treatment and inhibited cell proliferation

[5] X. Peng and R.G. Mehta, unpublished data.

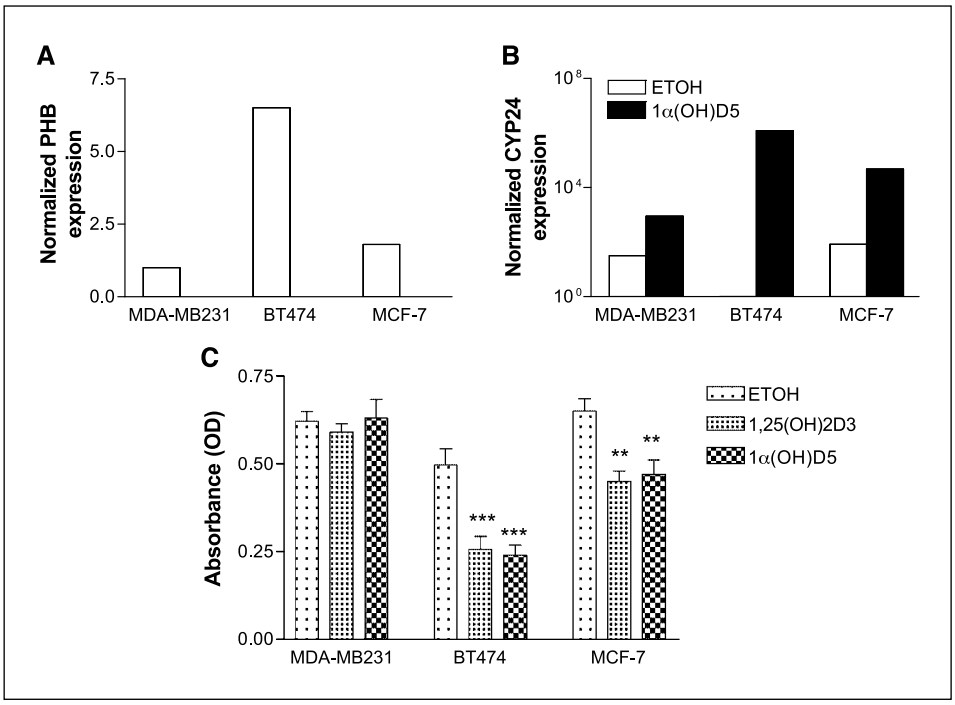

Figure 2. Correlation of prohibitin basal transcriptional sensitivity to vitamin D in breast cancer cells. *A,* basal transcriptional level of prohibitin in ER^+ and ER^- breast cancer cell lines. The basal prohibitin level in MDA-MB231 cells was set as 1. *Columns,* mean of two independent experiments with duplicate analyses of each RNA sample. *B,* transcriptional induction of CYP24 by $1\alpha(OH)D_5$ (0.5 μmol/L) in breast cancer cell lines analyzed by real-time RT-PCR. The basal CYP24 level in BT474 cells was set as 1. CYP24 mRNA level in each sample was normalized to β-actin and the basal level of BT474 cells. *Columns,* mean of two independent experiments with duplicate analyses of each RNA sample. *C,* effects of vitamin D analogues on cell proliferation as measured by MTT assay in breast cancer cells. Cells were incubated for 4 days with $1\alpha(OH)D_5$ (0.5 μmol/L) and $1,25(OH)_2D_3$ (100 nmol/L). ER^+ breast cancer cells (MCF-7 and BT474) are sensitive to vitamin D treatment, whereas ER^- breast cancer cells (MDA-MB231) are resistant to vitamin D treatment. *Columns,* mean of three independent experiments with eight wells per treatment in each experiment; *bars,* SE. **, $P < 0.01$, compared with control; ***, $P < 0.001$, compared with control.

by 50% after 4-day treatment in BT474 cells. MCF-7 cells are responsive to $1\alpha(OH)D_5$ treatment but less sensitive compared with BT474 cells, whereas MDA-MB231 cells were resistant to $1\alpha(OH)D_5$ treatment. $1\alpha(OH)D_5$ failed to inhibit cell proliferation and only slightly induced CYP24 expression in MDA-MB231 cells. These data show that basal expression level of prohibitin could potentially contribute to cellular sensitivity to vitamin D in breast cancer cells.

Prohibitin is localized to the nuclei in MCF-7 breast cancer cells. We examined the distribution of prohibitin and VDR in MCF-7 cells by fluorescent immunostaining. MCF-7 cells are well

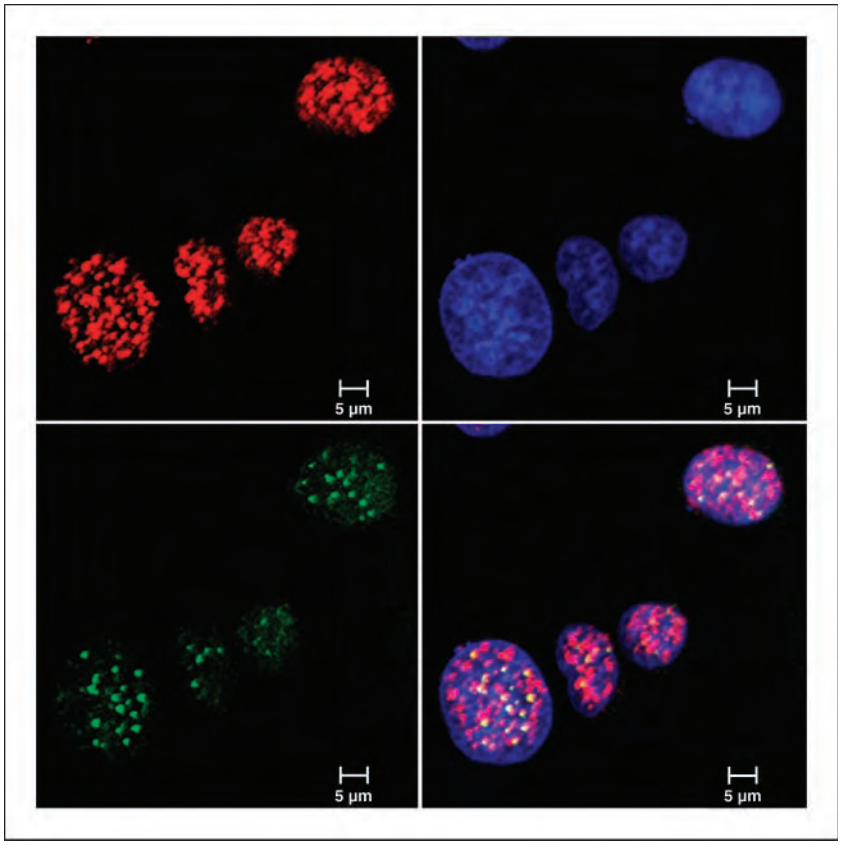

Figure 3. Localization of prohibitin and VDR in the nuclei of MCF-7 cells. Cultured MCF-7 cells were immunostained with rat anti-prohibitin (*top left, red*) and mouse anti-VDR (*bottom left, green*) mAbs. *Top right, blue,* nuclei were stained with DAPI. Fluorescent immunostaining of prohibitin (*red*) and VDR (*green*) in MCF-7 cells shows clear colocalization (*bottom right, yellow*) of VDR with a portion of prohibitin in the nuclei.

characterized, express moderate level of prohibitin (Fig. 2*A*), and used for cell transfection studies in our laboratory. Confocal microscopic analysis showed that prohibitin was localized in the nuclei and a portion of prohibitin was colocalized with VDR (Fig. 3). Such nuclear accumulation of prohibitin has been previously reported (11–13, 16). The colocalization of prohibitin and VDR suggested that a potential physical association between the two molecules could exist and that prohibitin could regulate VDR-mediated cellular responses.

Characterization of prohibitin expression and its role in response to vitamin D in MCF-7 prohibitin *Tet-On* model. Because prohibitin is a target gene of vitamin D and a direct physical association between prohibitin and VDR may occur, we hypothesized that prohibitin expression plays a role in response to vitamin D. To test this hypothesis, a MCF-7 cell line expressing tetracycline-inducible c-Myc-tagged prohibitin (prohibitin *Tet-On* model; ref. 11) was used. Using this model, we evaluated the effect of prohibitin overexpression on vitamin D–induced cellular response. We first confirmed the increased expression of prohibitin with tetracycline (1 μg/mL) and its derivative doxycycline (50 ng/mL), the two *Tet*-system inducers. After 24 hours of treatment, both inducers effectively induced exogenous prohibitin expression. The c-Myc-tagged prohibitin was separated with a molecular mass of ~34 kDa from the endogenous prohibitin (28-30 kDa; Fig. 4*A*). Because doxycycline is water soluble and the induction concentration is much lower than tetracycline, we further optimized the concentration of doxycycline used in this *Tet-On* system. The cells were treated with different concentrations of doxycycline, and exogenous prohibitin expression was analyzed by immunoblotting (Fig. 4*B*). Results showed that doxycycline induced dose-dependent prohibitin expression with maximum induction at the concentration of 20 ng/mL. This concentration of doxycycline was therefore used for all the later experiments. Because the exogenous prohibitin is c-Myc tagged, we used anti-c-Myc antibody to confirm that the ~34-kDa protein band is in fact prohibitin by immunoblotting. As shown in Fig. 4*C*, the doxycycline-induced ~34 kDa protein band was also detected by anti-c-Myc antibody, whereas in *Non-Tet-On* MCF-7 cells doxycycline treatment did not induce prohibitin expression. As expected, reprobing the membrane with anti-prohibitin antibody revealed that all endogenous and exogenous prohibitin were detected and that the anti-prohibitin antibody was specific to prohibitin without cross-reaction with other proteins (Fig. 4*D*). These results once again confirm the nuclear localization of prohibitin shown in Fig. 3. To detect the possible physical interaction between prohibitin and VDR, coimmunoprecipitation was done using cell lysates made from cultured MCF-7 *Tet-On* cells. As shown in Fig. 4*E*, after immunoprecipitating proteins with polyclonal anti-VDR and monoclonal anti-c-Myc antibodies, the precipitated complexes were subjected to Western blot analysis using anti-prohibitin antibody (Fig. 4*E*). The proposed physical interaction between prohibitin and VDR was not detectable (Fig. 4*E, lane 4*), whereas c-Myc antibody precipitated exogenous c-Myc-tagged prohibitin from cell lysates (Fig. 4*E, lane 6*). However, reprobing the membrane with VDR antibody once again failed to detect physical interaction between prohibitin and VDR (data not shown). Interestingly, as shown in Fig. 4*E* (*lane 6*), in addition to exogenous c-Myc prohibitin band, endogenous prohibitin (28-30 kDa) as well as multiple protein bands at 90 to 130 kDa were detected by prohibitin antibodies from c-Myc antibody-precipitated complexes.

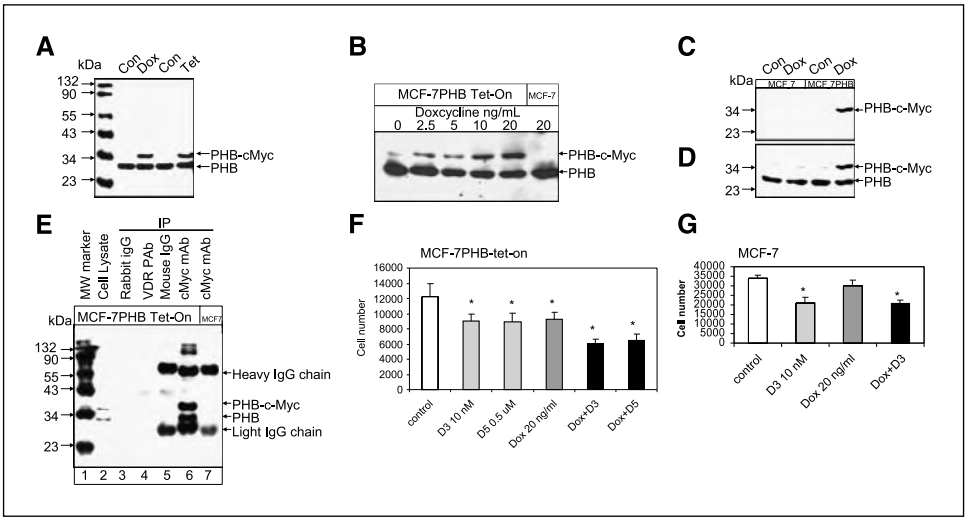

Figure 4. Characterization of prohibitin expression and cellular response to vitamin D in prohibitin *Tet-On* MCF-7 cell line. *A,* Western blot analysis of prohibitin expression in *Tet-On* MCF-7 cells expressing tetracycline-inducible c-Myc-tagged prohibitin. Both tetracycline (*Tet*; 1 μg/mL) and doxycycline (*Dox*; 50 ng/mL) induced exogenous prohibitin (~34 kDa) expression after 24 hours of treatment. *B,* optimization of doxycycline concentration in induction of prohibitin expression in *Tet-On* MCF-7 cells. Doxycycline (20 ng/mL) was determined to be the best concentration for this system. *C* and *D,* Western blot analyses of c-Myc-tagged prohibitin in *Tet-On* MCF-7 cells using anti-c-Myc (*C*) and anti-prohibitin (*D*) mAbs. Both antibodies specifically detect the exogenous prohibitin. *E,* immunoprecipitation (*IP*) of VDR (*lane 4*) and exogenous prohibitin (*lane 6*) from cell lysates made from MCF-7PHB *Tet-On* cells using anti-VDR polyclonal antibody (*VDR PAb*) and anti-c-Myc mAb. Prohibitin was not coprecipitated with VDR, but endogenous prohibitin was coprecipitated with exogenous c-Myc-tagged prohibitin (*lane 6*), indicating lack of physical interaction between VDR and prohibitin and the formation of prohibitin homomers. *Non-Tet-On* MCF-7 cells (*lane 7*) served as a negative control for c-Myc-tagged exogenous prohibitin. *F,* inhibition of cell proliferation and enhancement of the antiproliferative effect of vitamin D in MCF-7 cells by prohibitin overexpression. *Tet-On* MCF-7 cells were incubated with either 10 nmol/L $1,25(OH)_2D_3$ (*D3*) or 0.5 μmol/L $1\alpha(OH)D_5$ (*D5*) in the presence or absence of 20 ng/mL doxycycline for 4 days, and cell number was determined. *Columns,* mean of three independent experiments with triplicate wells in each experiment; *bars,* SE. *, $P < 0.05$, compared with the corresponding control [$1,25(OH)_2D_3$ versus control; $1\alpha(OH)D_5$ versus control; doxycycline versus control; doxycycline + $1,25(OH)_2D_3$ versus doxycycline or $1,25(OH)_2D_3$ only; doxycycline + $1\alpha(OH)D_5$ versus doxycycline or $1\alpha(OH)D_5$ only]. *G,* MCF-7 cells (*Non-Tet-On*) were grown in 12-well plates and treated with 10 nmol/L $1,25(OH)_2D_3$ in the presence or absence of doxycycline for 4 days. In *Non-Tet-On* MCF-7 cells, doxycycline at 20 ng/mL had very little effect on cell proliferation. *, $P < 0.05$, compared with the corresponding control [$1,25(OH)_2D_3$ versus control; doxycycline + $1,25(OH)_2D_3$ versus doxycycline only].

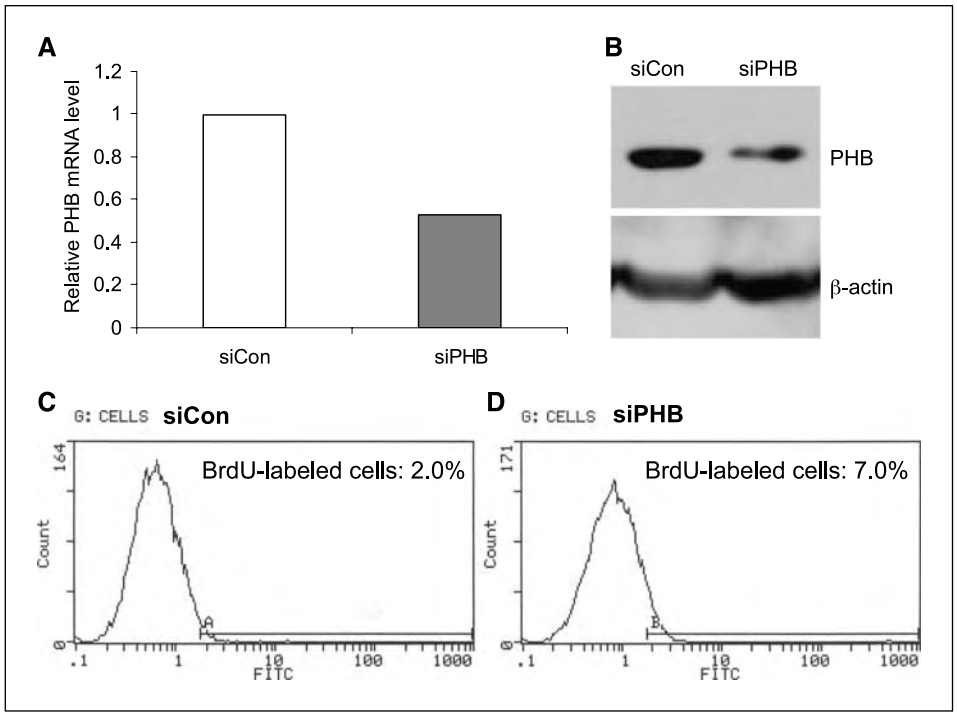

Figure 5. Correlation of silencing of prohibitin by RNAi with increased BrdUrd incorporation in MCF-7 cells. *A* and *B,* real-time RT-PCR (*A*) and Western blot (*B*) analysis of prohibitin expression after siRNA transfection, confirming knockdown of prohibitin at both mRNA and protein level. *C* and *D,* representative FACS analysis of BrdUrd incorporation in control siRNA–transfected (*siCon*; *C*) and prohibitin siRNA–transfected (*siPHB*; *D*) MCF-7 cells. Increased BrdUrd incorporation is observed after prohibitin knockdown. Experiments were repeated twice.

The nature of the protein bands at 90 to 130 kDa is not clear at this time. Coimmunoprecipitation of endogenous prohibitin showed the formation of prohibitin homomers. This is consistent with the large prohibitin spots (possibly prohibitin homomers) observed in the nuclei in Fig. 3. To test the effect of prohibitin expression on vitamin D–induced cellular response, the MCF-7 cells expressing tetracycline-induced prohibitin were treated with $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ in the presence and absence of doxycycline for 4 days and the cell number in each well was determined by direct counting (Fig. 4*F* and *G*). Results showed that induction of prohibitin expression using doxycycline significantly inhibited cell proliferation, indicating that prohibitin itself has antiproliferative effect, which is supported by previous reports (11). Incubation of *Tet-On* MCF-7 cells for 4 days with 0.5 μmol/L $1\alpha(OH)D_5$ or 10 nmol/L $1,25(OH)_2D_3$ effectively inhibited cell proliferation by ~27% in the absence of doxycycline, whereas vitamin D treatments in the presence of doxycycline (*Tet-On*, prohibitin is overexpressed) inhibited cell proliferation by ~50% in these cells. On the other hand, the incubation of *Non-Tet-On* MCF-7 cells with $1,25(OH)_2D_3$ in the presence of doxycycline had no additive inhibitory effect on cell proliferation, indicating that the additive effect of vitamin D treatment is associated with the overexpression of prohibitin. These results support the notion that prohibitin is involved in the antiproliferative effect of vitamin D and at least partially contributes to cellular sensitivity to vitamin D.

Silencing of prohibitin using RNAi increased BrdUrd incorporation and cell distribution in S phase of cell cycle in MCF-7 cells. Given that prohibitin is a target gene of vitamin D and that it contributes to the antiproliferative action of vitamin D, we investigated the effect of knockdown of prohibitin by RNAi on cell proliferation in MCF-7 cells. Cell proliferation was evaluated using BrdUrd incorporation assay and cell cycle analysis. Transfection for 24 hours followed by 48 hours of incubation with regular culture medium containing 5% FBS decreased prohibitin mRNA by 50% in prohibitin siRNA–transfected cells as measured by real-time RT-PCR (Fig. 5*A*) and was accompanied with decreased prohibitin protein expression by 80% as evaluated by Western blot analysis (Fig. 5*B*). BrdUrd incorporation assay using FACS analysis showed a significant increase (>3-fold) in BrdUrd-labeled cells after prohibitin knockdown (Fig. 5*C* and *D*) compared with nonsilencing control siRNA-transfected cell. Cell cycle analysis of prohibitin siRNA–transfected MCF-7 cells showed increased cell distribution in S phase and decreased cell distribution in G_1-G_0 phase of cell cycle (Table 1), confirming the results of BrdUrd incorporation assay after prohibitin is silenced by RNAi.

Prohibitin is not involved in CYP24 transactivation by vitamin D. Because prohibitin is involved in the antiproliferative action of vitamin D, we asked if prohibitin is involved in the CYP24 transactivation by vitamin D. *CYP24* is an established direct target gene of VDR and is also a very sensitive marker for vitamin D response. We initially hypothesized that prohibitin expression affects the CYP24 transcription expression if a direct physical interaction between prohibitin and VDR was present, even if it was not detectable by coimmunoprecipitation. We did real-time RT-PCR analysis of CYP24 transactivation by vitamin D in the

Table 1. Cell cycle effects of prohibitin silencing on MCF-7 cells

	G_1-G_0	S	G_2-M
Control siRNA	66.8 ± 2.1	31.7 ± 1.1	1.5 ± 0.4
Prohibitin siRNA	60.4 ± 1.9	36.5 ± 1.5	3.1 ± 0.7

NOTE: Cell cycle analysis of control siRNA–transfected and prohibitin siRNA–transfected MCF-7 cells. Cells were incubated with siRNA for 24 hours and then in fresh culture medium containing 5% FBS for another 48 hours. Values are mean ± SD of triplicate samples.

Tet-On MCF-7 cells (Fig. 6*A*) and prohibitin siRNA–transfected MCF-7 cells (Fig. 6*B*). In MCF-7 *Tet-On* model, cells were pretreated with or without doxycycline for 24 hours followed by treatment with $1,25(OH)_2D_3$ (10 nmol/L) for additional 24 hours. As shown in Fig. 6*A*, treatment with $1,25(OH)_2D_3$ dramatically induced CYP24 mRNA expression as expected; however, induction of prohibitin expression by pretreatment with doxycycline did not affect CYP24 mRNA expression induced by $1,25(OH)_2D_3$ (Fig. 6*A*). Similarly, silencing of prohibitin by siRNA in MCF-7 cells did not affect CYP24 transactivation induced by $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ (0.5 μmol/L; Fig. 6*B*), indicating that CYP24 transactivation by vitamin D in *Tet-On* MCF-7 cells and prohibitin siRNA–transfected MCF-7 cells was not affected by prohibitin expression level. To further confirm this, we also transiently transfected MDA-MB231 cells expressing low level of prohibitin (Fig. 2*A*) with prohibitin expression vector (pcDNA3.1PHB) to overexpress prohibitin in this cell line. After transfection, cells were treated with $1,25(OH)_2D_3$ for 24 hours. As shown in Fig. 6*C*, transient transfection caused dramatic overexpression of prohibitin as evaluated by real-time RT-PCR, but CYP24 transactivation by $1,25(OH)_2D_3$ was not significantly affected, supporting the results obtained from MCF-7 cells.

Discussion

VDR signaling pathway is fundamental to chemoprevention and therapy of breast cancer using vitamin D analogues. Although the antiproliferative effects of vitamin D in breast cancer cells are well recognized, the detailed signaling pathway and target genes involved in this process still need to be identified and characterized. In this study, we identified prohibitin as a novel target gene of VDR function, which is involved in the antiproliferative action of vitamin D.

Our initial *in silico* effort identified two potential VDR/RXR binding sites in the promoter region of prohibitin. Although whether VDR directly binds to the promoter of prohibitin still needs further experimental verification, our real-time RT-PCR and immunoblot analyses have confirmed that prohibitin is a primary vitamin D target gene. Many vitamin D target genes, including *osteopontin, osteocalcin, calbindin*, etc., which are classically involved in calcium homeostasis and bone metabolism, have been identified. In addition, a few cell cycle–regulated genes, such as *p21* and *cyclin C*, are also known to be regulated by vitamin D and related to the antiproliferative action of vitamin D. The identification of prohibitin as a novel vitamin D target gene adds a new member to the list of vitamin D–responding genes related to cell cycle control. The transcriptional and translational regulation of prohibitin by vitamin D is very similar to that of cyclin C (30, 31) in breast cancer cells. Interestingly, at mRNA level, both prohibitin and cyclin C are quickly up-regulated after vitamin D treatment with peaks at 8 and 3 hours after treatment, respectively, followed by gradual reduction in expression, whereas CYP24 induction lasts for at least 24 hours. At peak mRNA levels, both prohibitin and cyclin C are ~2-fold of their basal levels (30), whereas at protein level both are >2-fold of their basal levels (31). Such quick, transient, and significant regulation of prohibitin by vitamin D might be a sign of tight control of the expression of this gene in response to vitamin D.

Prohibitin has multiple functions ranging from a role in aging (32), epithelial cell migration (23), mitochondrial inheritance (33), and as a chaperone for the stabilization of mitochondrial proteins in yeast (9) to its role in cell cycle control, apoptosis (12), and as associated molecules in cell surface receptors in mammalian cells (34, 35). Accumulative evidence shows its nuclear function in transcription regulation. Our data show that a portion of prohibitin colocalizes with VDR in the nuclei of MCF-7 cells, and it is involved in VDR-mediated antiproliferative action of vitamin D. Prohibitin was found to interact with multiple molecules in the cells, including Bap37, Rb, p53, E2F, cRaf-1, α-actinin, and annexin A2 (36), and also form homomers with prohibitin itself (Fig. 4*E*). These

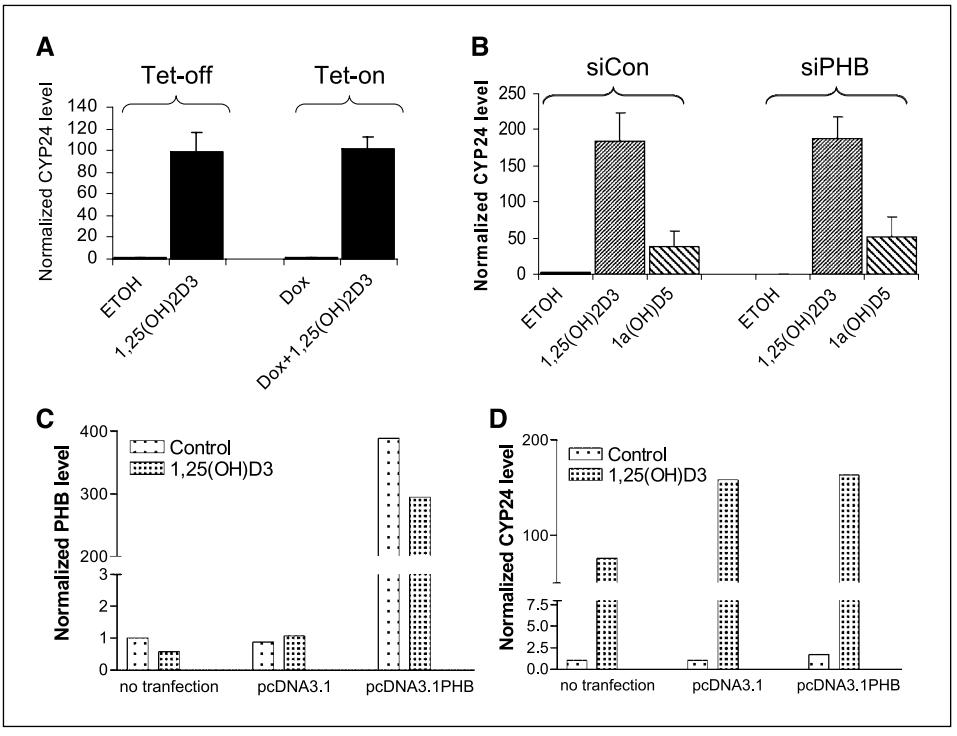

Figure 6. Effects of prohibitin expression on the CYP24 transactivation induced by vitamin D. *A,* real-time RT-PCR analysis of CYP24 transcription level after treatment of prohibitin *Tet-On* MCF-7 cells with $1,25(OH)_2D_3$ (10 nmol/L) in the presence (*Tet-On*) or absence (*Tet-Off*) of doxycycline (20 ng/mL). *Columns,* mean of three samples with duplicate analyses of each sample; *bars,* SE. *B,* knockdown of prohibitin by RNAi in MCF-7 cells did not affect CYP24 transactivation by vitamin D. *Columns,* mean of three independent experiments done in duplicate; *bars,* SE. Cells were treated for 48 hours with $1,25(OH)_2D_3$ (10 nmol/L) or $1\alpha(OH)D_5$ (0.5 μmol/L) after transfection of siRNA. *C* and *D,* prohibitin overexpression in MDA-MB231 cells does not affect CYP24 transactivation. MDA-MB231 cells were transiently transfected with prohibitin expression vector, and then cells were treated by $1,25(OH)_2D_3$ (10 nmol/L) for 24 hours. Prohibitin overexpression (*C*) and CYP24 transactivation (*D*) were measured using real-time RT-PCR. *Columns,* mean of two independent experiments.

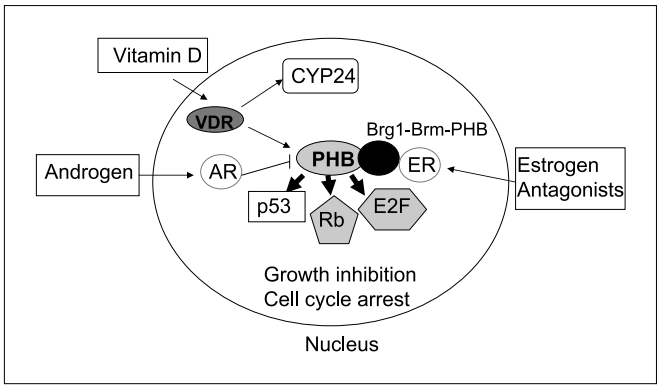

Figure 7. Schematic model to explain potential mechanism of action of nuclear prohibitin in cancer cells. Prohibitin is up-regulated by VDR-mediated action of vitamin D, which in turn inhibits cell proliferation. Action of estrogen antagonists mediated via ER also requires prohibitin for its antiproliferative action. On the other hand, prohibitin is down-regulated by androgen and in turn enhances cell proliferation. Thus, prohibitin might function as a target gene for steroid hormones and modulate the transcription by recruiting Brg-1/Brm. Prohibitin also interacts with E2F, p53, and pRb. These interactions ultimately result in the cell cycle arrest or growth inhibition. *AR,* androgen receptor.

results suggest that prohibitin may function as large complex homomers, heteromers, or multimers with other molecules regardless of its location. Because the primary sequence of prohibitin has predicted α-helical structures in its COOH-terminal end (37, 38), which forms the basis for interactions between prohibitin and other proteins, we initially proposed that prohibitin could physically interact with VDR and regulate VDR-mediated cellular response and placed much effort in identifying the interaction between the two molecules. However, we were not able to coprecipitate prohibitin with VDR directly from cell lysates using multiple breast epithelial cell lines and various experiment conditions, indicating that the affinity between VDR and prohibitin in the intact cells could be very low or they do not physically interact with each other in the cells. Later experiments on CYP24 transactivation by vitamin D further support this notion because *CYP24* is a very sensitive marker in response to vitamin D and a direct target gene of VDR. If prohibitin physically binds to VDR, the expression level of prohibitin would affect CYP24 transactivation by vitamin D. However, the results indicated that prohibitin-mediated vitamin D response on cell proliferation was independent of CYP24 transactivation.

The detailed mechanism by which prohibitin can induce antiproliferative and cell cycle regulatory activity, although intriguing, is yet to be established. There is even considerable controversy about the function of prohibitin localized in the nuclei. Because emerging data show the diversified localization and translocation, it is not surprising to see that prohibitin is associated with multiple functions in the cells. It is believed that prohibitin can inhibit cell proliferation by directly interacting with both pRb and p53 pathways, suggesting a mechanism dependent of the pRb and p53 pathways is involved in the antiproliferative effects of prohibitin (10). Recent evidence has shown that prohibitin recruits Brg-1 and Brm to E2F-responsive promoters and that this recruitment is required for the repression of E2F-mediated transcription by prohibitin (35, 39). Brg-1 and Brm are involved in chromatin remodeling and mediating hormone-dependent transcriptional activation by nuclear receptors (10), which could provide a common explanation for the diverse effects of nuclear prohibitin in different cells. In breast cancer cells, the depletion of prohibitin by antisense or siRNA strategies inhibits the growth-inhibitory effect of the antiestrogen 4-hydroxytamoxifen and ICI 182,780. Moreover, the transfection of a construct expressing residues 304 to 357 of E2F, which inhibits the interaction between prohibitin and E2F, blocked the antiestrogenic effect, suggesting an important role of prohibitin in the antiproliferative actions of estrogen antagonists (39). Similar mechanism may also be involved in the antiproliferative action of vitamin D. In the present study, we observed that prohibitin is up-regulated by vitamin D treatment and its level correlates with the cellular sensitivity to vitamin D. Overexpression of prohibitin enhanced the antiproliferative effect of vitamin D in MCF-7 cells, whereas knockdown of prohibitin was associated with increased proliferating cells in the whole-cell population. These results identify prohibitin as one of the important molecules involved in the antiproliferative action of vitamin D. Similarly and in consistence with our studies, in androgen-stimulated LNCap prostate cancer cells, prohibitin is down-regulated (13). Interestingly, vitamin D3 and its analogue have been reported to suppress the androgen-stimulated growth of mouse mammary carcinoma SC-3 cells (40) and block *in vitro* and *in vivo* androgen-stimulated prostate cell growth (41).

Although prohibitin is involved in the antiproliferative action of vitamin D, it is not involved in the CYP24 transactivation by vitamin D, suggesting that vitamin D could use different VDR-mediated pathways for its antiproliferative effects and CYP24 activation, respectively. Previous studies have also reported that the growth-inhibitory pathway of $1,25(OH)_2D_3$ could be selectively abrogated, whereas cells remained sensitive to direct $1,25(OH)_2D_3$ signaling (induction of CYP24; ref. 42). A proposed interaction of prohibitin with VDR and other steroid receptors is diagrammatically shown in Fig. 7.

This is the first report to indicate that prohibitin is involved in the antiproliferative action of vitamin D. Our results, together with the findings of others for the role of prohibitin in the action of androgen (13) and estrogen antagonists (39), suggest that prohibitin may regulate the action of steroid hormones.

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Hepatic Activation and Inactivation of Clinically-relevant Vitamin D Analogs and Prodrugs

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Abstract. *Like most pharmaceutical agents, vitamin D analogs are subject to hepatic metabolism by a variety of cytochrome P450 (CYP)-based systems. Metabolism can involve activation as well as inactivation of the vitamin D analog and one of the more successful families includes the 1α-hydroxyvitamin D prodrugs (1α-OH-D_2, 1α-OH-D_3, 1α-OH-D_4, 1α-OH-D_5), that all require a step of activation. Some of these prodrugs are in use or clinical trial because they have a therapeutic advantage over calcitriol. However, the nature of the activation of these molecules is poorly understood, particularly with regard to the CYP isoform involved. Various transfected CYPs and hepatic cell lines combined with tandem LC-MS analysis were used to investigate the metabolism of a spectrum of vitamin D analogs, including 1α-OH-Ds and the topical analog, calcipotriol. In the case of the 1α-OH-Ds, evidence was found of multiple sites of side-chain hydroxylation consistent with the generation of more than one active form. The potential involvement of CYP27A and other putative 25-hydroxylases in 1α-OH-D activation was also shown, as well as the potential for CYP24 activation and inactivation. In the case of calcipotriol, the respective roles of non-vitamin D-related CYPs and CYP24 in the catabolism of this anti-psoriatic drug were dissected out using cell lines with or without CYP24 expression, allowing us to demonstrate the potential contribution of CYP24 to "vitamin D resistance". The implications of hepatic metabolism in the context of other facets thought to play a role in the mechanism of action of anticancer and antiproliferative vitamin D analogs are discussed.*

The importance of the liver in the activation of vitamin D has long been recognized (1). Over the past decade, the nature of the cytochrome P450 (CYP) species involved in the 25-hydroxylation of vitamins D_2 and D_3 has become much clearer, even if it has not been fully resolved (2). One mitochondrial CYP and four microsomal CYPs have been implicated as potential vitamin D-25-hydroxylases, though it appears that CYP2R1 possesses many of the criteria to be identified as the physiologically-relevant enzyme (3), including a type of human vitamin D deficiency rickets associated with a mutant form of the CYP2R1 enzyme (4). These criteria are worth repeating:

i) Kinetic experiments suggest that there are at least two isoforms in the mitochondrial and endoplasmic reticulum (microsomal) compartments.

ii) The mitochondrial form is a high capacity, low affinity 'pharmacologically-relevant' enzyme while the microsomal form is a low capacity, high affinity "physiologically-relevant" enzyme.

iii) Together the two enzymes must explain the *in vivo* observations that 25-hydroxylase is weakly regulated and leads to side-chain hydroxylation of both vitamins D_2 and D_3.

There are claims that CYP27A1 has many of the properties ascribed to the mitochondrial enzyme (5), though strangely it will 24-hydroxylate vitamin D_2 but will not 25-hydroxylate it at micromolar substrate concentrations. Of the microsomal forms, only CYP2R1 meets all the criteria ascribed to the "physiologically-relevant" enzyme including the ability to 25-hydroxylate vitamin D_2. Comparisons of putative microsomal 25-hydroxylases have been published (6, 7), but no studies have demonstrated the high affinity nature of the enzyme.

Few studies of the hepatic metabolism of vitamin D analogs have been published. The principal exceptions to this statement are studies of the 1α-hydroxyvitamin D prodrugs including 1α-OH-D_3 (One-alpha; Leo Pharma, Denmark); 1α-OH-D_2 (Hectorol; Bone Care International, Madison, WI, USA); 1α-OH-D_4; 1α-OH-D_5 (IIT, Chicago IL, USA) (8). These compounds have proven to be highly

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successful in the treatment of defects of calcium homeostasis and hyperparathyroidism associated with chronic kidney disease (CKD). All of these 1α-hydroxylated analogs are presumed to be active by virtue of being metabolized *in vivo* to side-chain hydroxylated species analogous to 1α,25-$(OH)_2D_3$ in order to execute their therapeutic effects (8). The prodrug 1α-OH-D_5 (IIT) has been shown to act as a chemopreventative agent in mammary carcinogenesis models and has been approved for NCI trials in breast cancer patients (9). However, currently no metabolic studies of 1α-OH-D_5 have been published and, thus, *in vitro* metabolic studies using liver systems would seem to be justified. Furthermore, though *in vitro* studies of the metabolism of 1α-OH-D_3 and 1α-OH-D_2 have been carried out and have suggested that a variety of mitochondrial and microsomal enzymes can hydroxylate these prodrugs (Figure 1) (5-7), there have been no studies to determine if the newer isoforms (*e.g.*, CYP2R1) can side-chain hydroxylate 1α-hydroxylated prodrugs at physiological substrate concentrations.

Calcipotriol (MC-903) is a vitamin D analog well-studied metabolically *in vitro*. Sorensen and colleagues (10) established that various liver preparations are able to convert the 24-hydroxylated analog into unsaturated and saturated 24-ketones and this work was extended by Masuda *et al.* (11), who showed that this metabolic pathway was present in vitamin D-target cells and went further to give 23- and 24- hydroxylated products as well as side-chain-cleaved metabolites. One question not answered in earlier work was the nature of the enzyme(s) responsible for the metabolism of calcipotriol. Since calcipotriol is topically applied, the metabolism of the drug in keratinocytes would seem particularly relevant. To date, since the highly inducible CYP24 represents the only vitamin D-metabolizing CYP identified in vitamin D target cells it seems logical to believe that CYP24 is involved in calcipotriol metabolism, but this hypothesis remains untested and unproven. With the availability of newer tools such as isolated CYPs, newly-synthesized 1α-hydroxylated vitamin Ds and the powerful new LC-MS technology, the metabolic objectives of the current study were to explore:

i) The ability of CYP2R1 in a cell model *in situ* to metabolize a physiological concentration of [9,11-^{3}H]1α-OH-D_2.

ii) The liver-cell mediated metabolism of 1α-OH-D_5 using newer LC-MS analytical procedures (11).

iii) The putative CYP24-mediated metabolism of calcipotriol.

Materials and Methods

Vitamin D analogs. Synthetic standard 1α-OH-D_3 and 1α,25-$(OH)_2D_3$ were provided by Leo Pharma (Ballerup, Denmark), 1α-OH-D_2 and [9,11-^{3}H]1α-OH-D_2 (35 Ci/mmol) by Bone Care International and 1α-OH-D_5 by IIT Research Institute (Chicago, IL, USA).

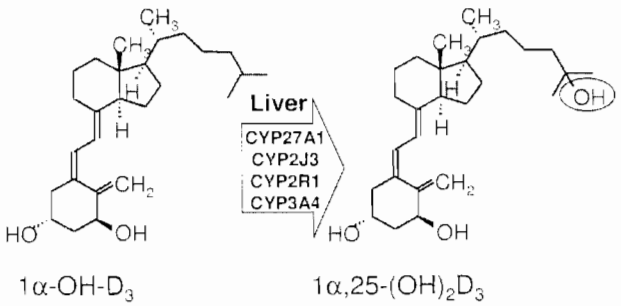

Figure 1. *Enzymatic activation of 1α-hydroxylated vitamin D prodrugs by liver enzymes. The putative cytochrome P450 species are indicated by the letter codes. All CYPs, except for the mitochondrial CYP27A1, are microsomal.*

Cell culture. HepG2 and V79-4 were obtained from ATCC (Manassas,VA, USA) and V79-hCYP24 cells have been described elsewhere (5, 12). Vectors containing mCYP2R1 and hCYP2R1 (13) were kindly provided by Drs. David W. Russell and David Mangelsdorf (Department of Molecular Genetics, University of Texas Southwestern Medical Center Dallas, TX, USA). The V79-4 cells ($1.5x10^5$ cells) were transiently transfected with 0.2 μg of pCMV6-mCYP2R1 or pCMV6-hCYP2R1 in the presence of 10 μl lipofectamine and 10 μl Plus reagents (Invitrogen) in serum-free DMEM for 3 h at 37°C. Cultured or transfected cells were cultured to confluence in FCS-containing medium and then incubated with vitamin D substrates, in the absence of FCS but containing 1% bovine serum albumin, for the indicated time period.

Vitamin D analysis. HPLC was carried out using Waters Alliance 2695 separations module, a photo-diode array detector and a Berthold on-line radioactivity detector, as described previously (12). In the 1α-OH-D_5 metabolic studies, metabolites were analyzed by LC-MS (Micromass Ultima) in ES+ scanning mode over a m/z range of 50-600, using a methanol/water-based gradient system on Zorbax SB-C18 at 200 μl/min over 30 min. Metabolites of 1α-OH-D_5 were identified on the basis of their characteristic molecular ions, dehydration products and other adducts after comparison to the side-chain metabolites of 1α-OH-D_3 produced in the same cell line.

Results

Enzymatic properties of CYP2R1 towards [9,11-^{3}H]1α-OH-D_2. Paramount in the proposed criteria to discriminate between "physiologically-relevant" 25-hydroxylases and those CYP enzymes that will simply perform this reaction *in vitro* under super-physiological conditions of high substrate concentration, is the experiment to explore if a putative 25-hydroxylase will 25-hydroxylate a vitamin D_2 substrate at nanomolar concentration. The availability of high specific activity radiolabelled [9,11-^{3}H]1α-OH-D_2 allowed us to test CYP2R1 for this property using transient transfection in a host V79 cell devoid of basal activity. Figure 2 shows the results of experiments using either mouse or human enzyme

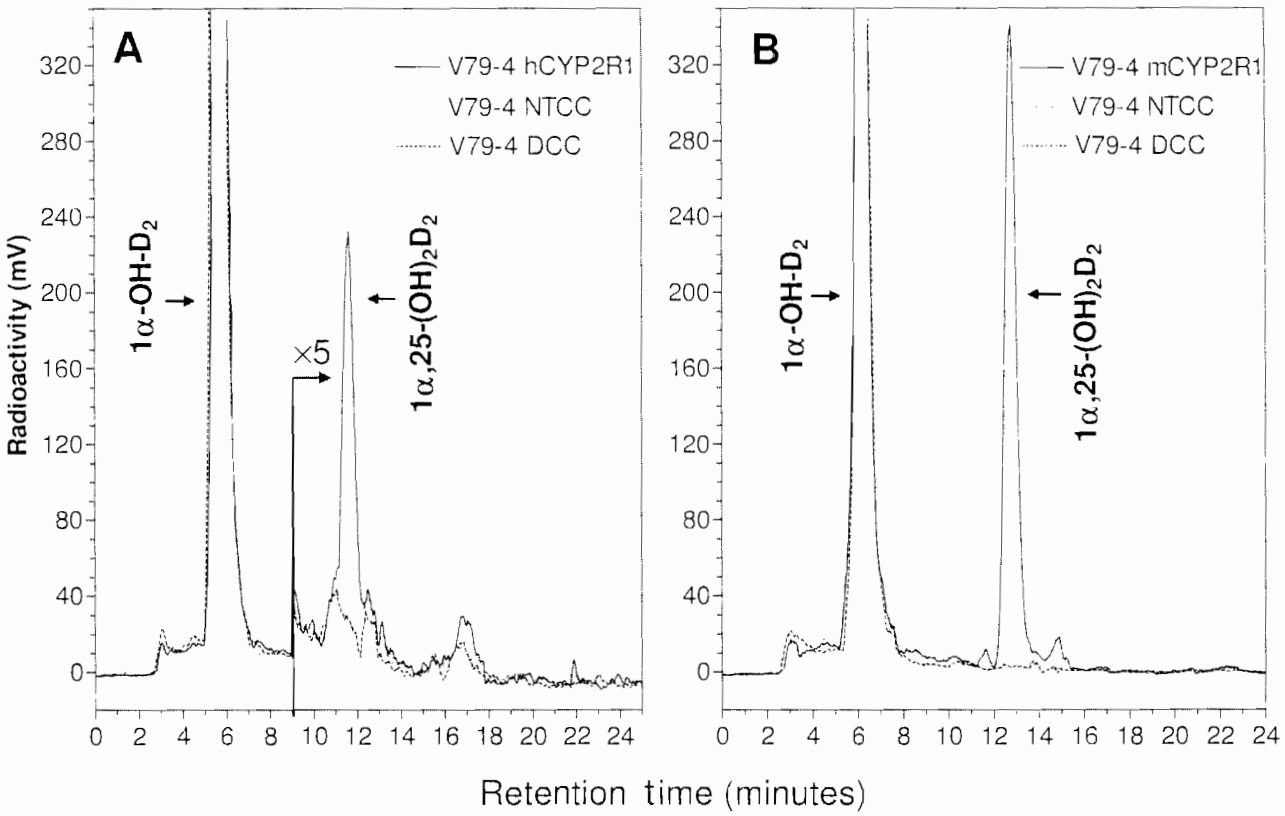

Figure 2. *Transfection studies of (A) human CYP2R1 and (B) mouse CYP2R1. Vectors containing the CYPs under investigation were transiently transfected in V79-4 host cells and then incubated with 10 nM [9,11-^{3}H]1α-OH-D_2 for 24 h. HPLC of the lipid extracts using an on-line radioactivity detector gave the traces shown. Standard 1α,25-$(OH)_2D_2$ co-migrated with the radioactive peak in each case. Note that the slight shifts in the retention times of both substrate and product in panels (A) and (B) were due to day-to-day variability in column conditions. NTCC=non-transfected cell control; DCC=dead cell control.*

incubated with 10 nM [9,11-^{3}H]1α-OH-D_2 for 24 h. In the chromatogram traces depicted, no products were formed in control extracts (parental V79 cells or dead-cell controls) while, after the expression of mouse or human CYP2R1, an additional peak appeared running between 12-13 min in each trace and co-migrating exactly with 1α,25-$(OH)_2D_2$. This peak was absent when CYP27A1 was transfected into the same cells (data not shown).

Metabolites of 1α-OH-D_5 from HepG2 cells evaluated using LC-MS-based analytical procedures. When 1α-OH-D_5 (10 μM) is incubated with HepG2 cells for 24-48 h a number of putative hydroxylated products are produced which elute chromatographically more slowly than the substrate and close to 1α,25-$(OH)_2D_3$ on straight-phase HPLC. Compared with control incubations using the same cells and 1α-OH-D_3 (10 μM), where there is a demonstrable production of 1α,25-$(OH)_2D_3$ and 1α,26-$(OH)_2D_3$, the yield of metabolites from 1α-OH-D_5 was disappointingly low, suggesting that the rate of activation in the liver was much less efficient for 1α-OH-D_5 than for 1α-OH-D_3. On reverse-phase HPLC, hydroxylated metabolites of 1α-OH-D_5 ran more polar than the parent molecule but less polar than 1α,25-$(OH)_2D_3$. On LC-MS (Figure 3), these metabolites all showed spectra consistent with them being mono-hydroxylated products with MH^+ ions of m/z 445 (MWt=444; MWt of parent 1α-OH-D_5=428). In comparison, the LC-MS of 1α,25-$(OH)_2D_3$ derived from 1α-OH-D_3 is also contrasted with its parent molecule in Figure 3. Though the mono-hydroxylated products of 1α-OH-D_5 are likely to be hydroxylated in the side-chain, LC-MS/MS analysis, and possibly other technologies are required to pinpoint the exact site of this hydroxylation. These studies are on-going, but the current experiments have established that hepatoma cells are able to metabolize the 1α-OH-D_5 substrate to hydroxylated products, albeit less efficiently than 1α-OH-D_3.

Metabolism of calcipotriol(MC-903) by V79-hCYP24 cells. Calcipotriol metabolism can be readily followed in both hepatoma and keratinocyte cell models, suggesting that this cyclopropane ring-containing analog is subject to the same type of modification at the C-24 position that is observed

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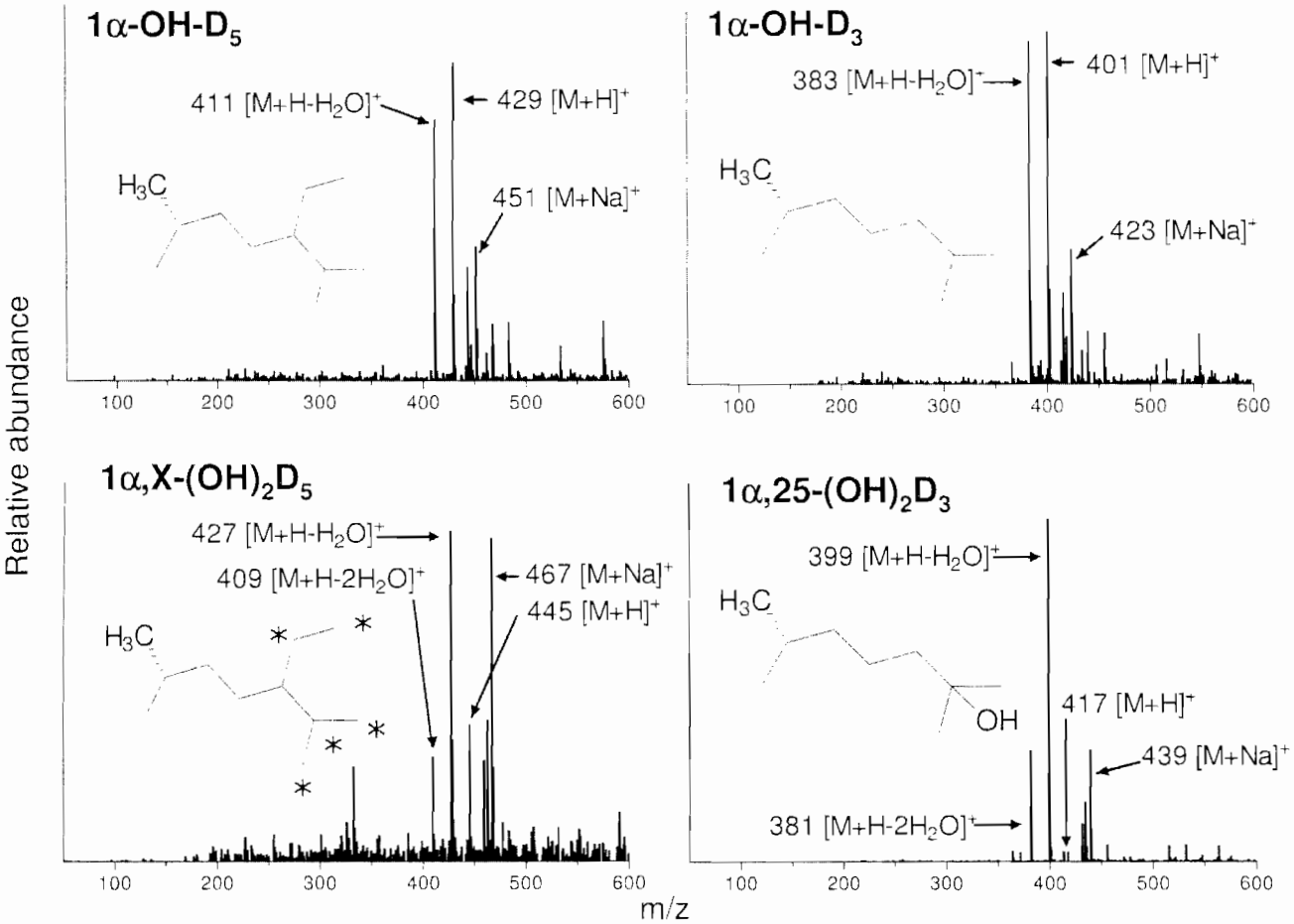

Figure 3. *LC-MS of specific isolated metabolic products of 1α-OH-D_3 and 1α-OH-D_5 produced by HepG2 cells along with their respective substrates. MS1 spectra were produced in electrospray positive (ES+) mode using a Micromass Quattro Ultima triple-quadrapole instrument in MS1 mode. Metabolites were generated by incubating 1α-OH-D_3 or 1α-OH-D_5 with HepG2 cells and the products isolated using preparative LC on Zorbax-SIL. Mass spectra were performed on-line using a reverse-phase LC step. ES+ spectra showed the usual MH+ ions, their dehydration products and various adducts of the metabolites. Asterisks on the 1α-OH-D_5 molecule are putative hydroxylation sites targeted by hepatic CYPs.*

in broken cell systems and *in vivo* and described previously (10, 11). The studies performed here represent the first attempt to use an individual CYP, expressed in a stable manner in a Chinese hamster lung V79 host cell, to study its potential involvement in the metabolism of calcipotriol. In Figure 4, it is important to note that the host cell showed virtually undetectable metabolism without the introduction of the hCYP24 (Panel A). It is possible that the trace amount of Peak 3 in the chromatogram was incorrectly identified as the metabolite simply co-migrates with the saturated ketone of MC903 depicted in the inset of Figure 4A. This possibility is rendered more plausible considering that there was no evidence of the formation of its precursor, the unsaturated ketone (Peak 2 of Figure 4A). Another trivial explanation is that there was a minor amount of native, inducible CYP24 in the host V79 cells, although this was not observed when the experiments were performed with [1β-^{3}H]1α,25-$(OH)_2D_3$. In any event, the ability of V79-4 to generate metabolites was extremely weak. In contrast, V79-hCYP24 cells showed a robust production of a full spectrum of calcipotriol metabolites, observed previously in liver and keratinocyte models (10, 11). While it was believed possible that CYP24 might be responsible for the production of C-24 ketone Peak 3, the subsequent 23-hydroxylated products (Peak 4; actually a mixture of 4a and 4b) and side-chain cleaved products, there was no firm evidence to prove this. Though the data suggest CYP24 may be responsible for the saturation of the $\Delta^{22\text{-}23}$ in the side-chain of calcipotriol, these studies cannot exclude the fact that another enzyme is responsible and is unmasked by the production of the unsaturated ketone (Peak 2 of Figure 4B) by CYP24.

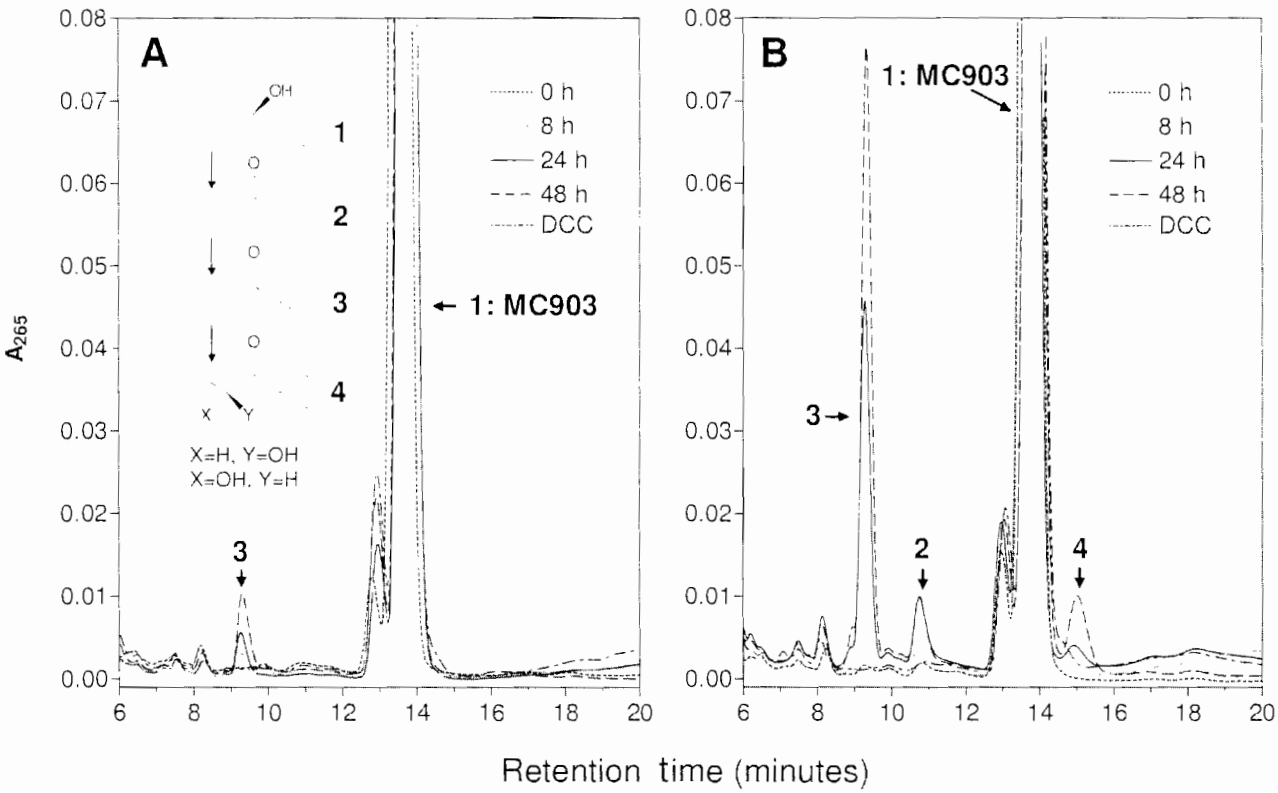

Figure 4. *Lipidomic studies of the metabolites of calcipotriol produced by (A) V79-4 cells and (B) V79-hCYP24 over a 48-h incubation period. Cells were incubated with calcipotriol (10 μM) for incubation times from 0-48 h and the lipid extracts run on HPLC using a photo-diode array detector. Chromatograms show A_{265nm} traces but putative hCYP24-generated metabolite peaks were checked for their vitamin D chromophore. Only peaks marked #1-4 in the traces possessed the characteristic vitamin D chromophore. Calcipotriol was Peak 1 running at 13.5-14 min and the shoulder just preceding at 13 min was pre-vitamin D. The structures of putative metabolites marked Peaks 2, 3 and 4 are shown in Panel (A).*

Discussion

The availability of expression systems which contain specific, cloned cytochrome 450s has opened up the possibility of dissecting out the role of individual enzymes in the metabolism of vitamin D and its analogs. Nowhere is this truer than with the subset of CYPs found in the liver and believed to be primarily involved in activation of the parent molecule and its prodrug analogs, but the approach can also be used for target cell CYPs involved in the metabolism of calcitriol analogs. Here the usefulness of the single CYP approach was demonstrated by showing that CYP2R1 was capable of the 25-hydroxylation of 1α-OH-D_2 at nanomolar concentrations and that CYP24 was responsible for some (if not all) of the steps of calcipotriol metabolism. Though, the approaches used entirely different substrate concentrations and, therefore, different modes of detection, the results were equally definitive.

The finding that CYP2R1 will 25-hydroxylate [9,11-^{3}H]1α-OH-D_2 at nanomolar concentrations makes this isoform unique and, taken together with the assertion that CYP2R1 is mutated in a patient with a form of hereditary rickets (4), and the distribution and substrate specificity studies produced by Russell's group (13) and others (7) suggest that it must be the 'physiologically-relevant' microsomal form of the 25-hydroxylase. It remains to be seen if others can demonstrate the relevance of CYP2J3 and CYP3A4 to vitamin D physiology, while for the mitochondrial form, CYP27A, the most generous view of its role in vitamin D homeostasis is that it represents a "pharmacologically-relevant" 25-hydroxylase enzyme. Though 1α-OH-D_2 (Hectorol) is marketed for treatment of patients with secondary hyperparathyroidism associated with CKD, this analog has potential in the treatment of hyperproliferative conditions. Indeed, one of its activated metabolites, 1α,24-$(OH)_2D_2$, formed by the action of CYP27A1 and CYP3A4, has been used to regulate the growth of prostate cancer cells *in vivo* and *in vitro* (14). The finding that there are also several enzymes in the microsomal fraction that will activate 1α-OH-D_2 to 1α,25-$(OH)_2D_2$, and that CYP2R1 is one of them, is valuable new information which opens up the possibility that the prodrug

can be activated and have growth inhibitory activity in extra-hepatic sites, since CYP2R1 is known to be more widely distributed than in the liver alone (13).

The other finding that CYP24 was involved in calcipotriol metabolism is novel and relevant to those using calcipotriol for the treatment of psoriasis. Clinical reviews of the use of topical calcipotriol report that approximately 70% of mild to moderate psoriasis patients respond positively to treatment (15). Whether these patients develop a resistance to calcipotriol or whether there are other mechanisms to explain the lack of responsiveness to calcipotriol in 30% of patients remains unclear. It is well known that the CYP24 gene is highly inducible by vitamin D agonists (16), so it is interesting to speculate that tissues exposed to such agents build up a resistance to the actions of the vitamin D analogs by CYP24 induction. Others have produced human tumor staging data to support the idea that CYP24 is a potential tumor suppressor gene which presumably acts by lowering the $1\alpha,25\text{-}(OH)_2D_3$ levels in cancer cells (17). Thus, the demonstration that CYP24 is involved in the catabolism of calcipotriol would suggest that patients who exhibit elevated CYP24 in responding tissues might show short-term improvement or fail to respond altogether. A test of this argument might be the use of a CYP24 inhibitor combined with a vitamin D agonist in "resistant" or non-responsive patients. Such a strategy is not dissimilar to that currently in advanced clinical trials by Cytochroma Inc. (Markham, Canada). The finding that CYP24 might be responsible for the saturation of the $\Delta^{22\text{-}23}$ in the side-chain of calcipotriol is reminiscent of our work (18) using $1\alpha,24\text{-}(OH)_2D_2$ to produce saturated, side-chain-cleaved metabolites when incubated with the CYP24-containing keratinocyte HPK1A-ras. The success of our studies suggests that V79-CYP24 cell assays (with the V79 control cells) could be used to determine the potential role of CYP24 in the catabolism of all calcitriol analogs in a routine manner, along with the usual battery of hepatic CYP assays commonly employed during pharmacology testing

The study of $1\alpha\text{-OH-}D_5$ by HepG2 cells provided the first positive evidence that the addition of the C24-C29 ethyl group in the side-chain of $1\alpha\text{-OH-}D_5$ does not block hepatic cell activation of the prodrug. Though the efficiency of $1\alpha\text{-OH-}D_5$ modification was certainly reduced over that observed in the control cultures with $1\alpha\text{-OH-}D_3$, measurable amounts of hydroxylated products were still formed. To date, these products have only been partially identified as mono-hydroxylated metabolites of $1\alpha\text{-OH-}D_5$. A more definitive identification is impossible because of the lack of useful fragments in the MS1 mode. Further information should be available after the same compounds have been subjected to the MS2 mode of LC-MS/MS. It should be noted that, while LC-MS/MS is a valuable and convenient addition to the armamentarium of the vitamin D analyst, it shows a disappointing inability to distinguish between isomers of vitamin D. In this respect, it is markedly inferior to its more laborious but more structurally-informative sister technique of GC-MS (19). In the case such as here, where no synthetic hydroxylated derivatives of $1\alpha\text{-OH-}D_5$ are currently available to be compared with unknown metabolites, the analyst is unable to assess LC retention times and has no definitive fragments to base identification upon. Consequently, the identification is equivocal. Nevertheless, the number (three) of distinct metabolite peaks in the extracts of liver-derived cells is less than the number (seven) of possible hydroxylation sites in the side-chain of $1\alpha\text{-OH-}D_5$ (See Figure 3, lower left panel), our work suggests that the enzymes involved generated a finite number of isomers. Based upon our experience with liver cells and $1\alpha\text{-OH-}D_3$, we would expect one of these products to be $1\alpha,25\text{-}(OH)_2D_5$, though $1\alpha,26\text{-}(OH)_2D_5$ or $1\alpha,29\text{-}(OH)_2D_5$ are other possibilities (20, 21).

Overall, the studies reported here demonstrate the versatility of the CYP complement of the liver to metabolize any synthetic vitamin D analog made by pharmaceutical chemists and indicates the complexity of the task of making effective anticancer vitamin D drugs that cause growth arrest in cancer cells before they are inactivated by CYPs.

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REVIEW

Clinical Trials Using Chemopreventive Vitamin D Analogs in Breast Cancer

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ABSTRACT

This article comprehensively reviews the clinical trials and considers the future directions of the use of vitamin D and its analogs in the treatment or chemoprevention of breast cancer. Chemopreventive treatment strategies strive to delay the onset of certain cancers, prevent the progression of malignant disease after diagnosis, or delay the advent of recurrence after curative treatment. We first summarize the epidemiological evidence that led to the hypothesis that vitamin D may have an anti-cancer activity. Vitamin D shows great potential as a therapy for breast cancer; however, its use in clinical trials has been hindered by the induction of hypercalcemia at a concentration required to suppress cancer cell proliferation. This has led to the development of less calcemic analogs of vitamin D. We review the clinical trials with breast cancer patients using vitamin D analogs. (*Cancer J 2006;12:445–450*)

KEY WORDS

Breast cancer, vitamin D, chemoprevention

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SEARCH STRATEGY AND SELECTION CRITERIA

Data for this review were identified by searches of PubMed, the Cochrane Library, Biosis, and references from relevant articles, using the search terms "vitamin D," "breast cancer," "chemoprevention," and "vitamin D analog." Abstracts from recent international meetings were also reviewed but were included only when they were the only known reference to the clinical trial or the research mentioned. Only papers published in English were included.

Breast cancer, the strongest risk factors for which include gender, age, and country of birth, continues to be a significant source of morbidity and mortality for women. Other primary risk factors for breast cancer are related to the female reproductive cycle and include age at menarche, nulliparity, age at first birth and duration of lactation, and age at menopause. Additional risk factors include exogenous estrogens, radiation exposure, alcohol consumption, and higher income and educational level.[1] Interestingly, location of residence has also been cited as a risk factor for breast cancer, which combines the two previously cited risk factors of radiation exposure and country of birth.[2] In the United States, the American Cancer Society estimates that 211,240 women are likely to be diagnosed with breast cancer in 2005 and 40,410 will die from their disease, making it the cancer with the greatest incidence in the United States and the second highest mortality, after lung cancer.[3]

Chemoprevention is an intervention in the carcinogenic process, possibly by a synthetic compound, which blocks, arrests, or reverses the progression of cancer.[4,5] Age is the most significant risk factor for many cancers, and awareness of this fact is a driving force behind research in cancer chemoprevention. With life expectancy continuing to rise in the general population, the incidence of breast cancer is likely to increase in the coming years. A large proportion of

women diagnosed with this disease can expect to experience significant morbidity during the course of their illness and the associated treatments. Chemopreventive treatment strategies strive to delay the onset of certain cancers, or prevent the progression of malignant disease after diagnosis, or delay the advent of recurrence after curative treatment. Initiatives using safe chemopreventive agents that are directed toward these tasks would be greatly welcome and are likely to have a major impact on women's health. Initial patient recruitment for breast cancer chemoprevention trials, however, is likely to be focused on patient groups with the specific high-risk factors alluded to earlier.

Vitamin D deficiency is common in the elderly.[7] Aging also lowers the ultraviolet radiation-mediated production of cholecalciferol in the skin. Moreover, estrogen deficiency, which primarily affects postmenopausal women, decreases the metabolic activation of vitamin D, as well as the expression of the vitamin D receptor (VDR).[8] VDRs are known to be expressed in a variety of cancer cells. Specific VDR polymorphisms can increase susceptibility to breast cancer, and women with certain genotypic variations may also be burdened with a more aggressive form of the disease, especially if the cancer metastasizes.[9] In addition, vitamin D deficiency per se may contribute to the incidence and mortality of breast cancer, and vitamin D deficiency prevention may thus be possible through increased sunlight exposure, improved diet, and supplemental vitamin D. Several studies measuring solar radiation have supported its beneficial role in breast and other cancers through its mediation of vitamin D synthesis, providing support for the hypothesis that vitamin D may provide some degree of protection against cancer. Epidemiologists estimate that perhaps 30% to 60% of all cancers could be avoided by modifications in diet,[10] and vitamin D is ingested in the diet, as well as synthesized through skin exposure to solar radiation. One potential chemopreventive agent for breast cancer that is currently being developed at our institutions is $1\alpha(OH)D_5$, or vitamin D_5, a synthetic analog of vitamin D. The effects of this analog will be investigated in two clinical trials, one involving breast cancer patients and the other with prostate cancer patients.[6]

VITAMIN D

Vitamin D was discovered by Edward Mellanby in 1919 in his experiments using dogs that were exclusively raised indoors, without exposure to sunlight or ultraviolet light.[11] Subsequently, E.V. McCollum was able to differentiate between the fat soluble vitamins A and D.[12] Vitamin D is a steroid hormone that has been shown to have antiproliferative and anti-tumor properties, making it a strong candidate for chemoprevention in breast or other malignancies. However, the usefulness of vitamin D in pharmacologic doses or over long periods of time has been limited because it can cause life-threatening hypercalcemia. For this reason, many new analogs that demonstrate less calcemic activity than vitamin D have been developed and some of these are being tested in phase I and phase II trials. Several recent reviews have also addressed the anti-cancer effects of vitamin D on breast cancer cells.[13,14]

A paper by Bertone-Johnson et al has suggested that vitamin D may be modestly beneficial for management of breast disease.[15] These researchers examined the relationship between stored plasma levels of 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)2D] and risk of breast cancer in a case-control study nested within the Nurses' Health Study cohort. Breast cancer cases had a lower mean serum 25(OH)D level in comparison to matched controls. This association was stronger in women aged 60 years and older.

VITAMIN D AND CANCER RISK

That adequate vitamin D intake may prevent the development of certain diseases—such as rickets, osteoporosis, tuberculosis, and even specific types of cancer—has been well documented.[16–20] The initial evidence suggesting an association between vitamin D and cancer protection was primarily epidemiologic in nature. Peller, for instance, observed that in occupations and environments where skin cancer rates were higher, the rates for other cancers were lower.[21] Subsequently, Apperly also reported that populations living farther from the equator had higher overall cancer death rates compared to those living closer to the equator, suggesting that increased sun exposure—and with it increased synthesis of vitamin D—led to decreased cancer-associated mortality.[22]

Historically, breast cancer mortality rates among American women have varied geographically and longitudinally, with the highest mortality occurring in the Northeast and the lowest mortality being reported in the South,[2,19,23,24] suggesting that solar radiation, which leads to vitamin D synthesis, might be protective against breast cancer.[25] Breast cancer mortality is also increased in cities compared to rural areas,[2] apparently because people living in urban areas may receive less sunlight exposure than those in rural areas at the same latitude, owing to air pollution. For instance, an analysis of data from a national cohort

NHANES I Epidemiologic Follow-up Study found that among women living in areas of high solar radiation, sunlight exposure, and adequate dietary vitamin D intake were associated with a 25% to 65% reduction in breast cancer risk.[24] Gorham et al also have shown statistically significant positive associations between acid haze air pollution, which blocks ultraviolet-B light, and age-adjusted breast and colon cancer mortality rates in a study covering 20 Canadian cities.[17] They hypothesized that the populations in such cities with high levels of acid haze may have been encumbered with vitamin D deficiencies. In addition, a similar ecological study in the former Soviet Union by Gorham et al also found a pattern of increased breast cancer incidence in those regions experiencing low sunlight levels.[26]

These geographic variations in which breast cancer mortality is inversely proportional to the intensity of the local sunlight have also been duplicated in the United States.[19] More recent studies have found that exposure to sunlight was inversely associated with mortality from breast cancer,[27] as was UV-BH radiation exposure per se.[28] Other investigations have also suggested this epidemiologic link between vitamin D and breast cancer.[29–31] The most likely mechanism by which sunlight exposure could inhibit the development of breast cancer is through the production of vitamin D. Casual exposure to sunlight remains one of the primary sources of vitamin D for women in the United States, and is, along with diet, a modifiable lifestyle factor.

A few studies contradict these findings. For example, Hiatt et al identified no relationship between elevated prediagnostic serum levels of $1,25(OH)_2D$ and the later diagnosis of breast cancer. However, the serum levels of vitamin D in this study were obtained an average of 15 years prior to the actual diagnosis of cancer, leaving unanswered the question whether elevated vitamin D could have a protective effect at a time closer to the clinically evident breast cancer.[32]

A single Canadian case control study evaluating dietary histories also did not identify an association between low vitamin D consumption and breast cancer development in women.[33] Indeed, breast cancer patients were found to have had a higher consumption of vitamin D than comparable controls. This study, however, did not consider the sunlight exposure-induced synthesis of vitamin D in these subjects.

Another study, examining incidence of breast cancer rather than mortality, also found little evidence of regional variation in breast cancer incidence rates.[34] Sturgeon et al, however, argued that the historically higher breast cancer mortality rates reported in the North are declining. Women in the Northeast are now experiencing a faster rate of decline in breast cancer mortality than their counterparts in the South, especially in specific groups such as black women of all ages and white women aged 20–49 years.[23]

Likewise, a study in Norway did not identify a negative association between cancer incidence and mortality and geographical latitude.[35] However, the investigators did point out that cases of breast, colon, and prostate cancer diagnosed in the summer and fall—the seasons when serum levels of vitamin D_3 are expected to be the highest—had a significantly better prognosis relative to the cases diagnosed during the winter months. Thus, vitamin D may have a beneficial effect on cancer-specific mortality, and supplemental vitamin D intake may improve cancer-related outcomes.

CLINICAL TRIALS WITH VITAMIN D OR ITS ANALOGS

There have been only a few breast cancer clinical trials with vitamin D or one of its analogs; these are reported in Table 1. In contrast to prostate cancer, such investigations in clinical trials are not as advanced (see Vijayakumar et al for a summary of clinical trials with prostate cancer patients and vitamin D analogs[36]).

To the best of our knowledge, the first study involved the use of topically applied calcipotriol. This vitamin D analog, also known as compound MC903, was used in the treatment of advanced breast cancer.[37] Treatment was administered to 19 patients with locally advanced or cutaneous metastatic breast cancer, with selected cancer nodules receiving the topically applied calcipotriol in doses of 100 micrograms daily. Five patients had to be withdrawn from the study before completion of the treatment; two of them because they developed hypercalcemia. The response rate too was low, with improvements noted in only 3 of the 14 patients who completed the 6 weeks of treatment (these 3 showed a 50% reduction in the bidimensional diameter of treated lesions). Of the remaining 14 patients, 5 unfortunately experienced progression of their disease, 5 reportedly had no change in their disease, and one had only a minimal response. Vitamin D receptors (VDR) were identifiable in the breast cancer cells of 7 patients, including all 4 who had had some response to the topical treatment. These data with calcipotriol suggested that this vitamin D analog may function through a mechanism involving the VDR.

Gulliford et al conducted a phase I trial to evaluate the maximum tolerated dose of another vitamin D analog, EB 1089 (Seocalcitol), in 36 patients with

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TABLE 1 Studies with Breast Cancer Patients and Vitamin D Analog Therapy

Study	No. of Patients	Therapy	Dose/Frequency	Duration
Bower 1991[37]	19	Calcipotriol ointment	100 μg, QD	6 weeks
Gulliford 1998[45]	36	EB 1089	0.15–17.0 μg/m^2 QD	1.5–33.5 weeks (10–234 days)
The Women's Health Initiative Study Group 1998[39]	45,000 women without breast cancer	Calcium and vitamin D_3	1000 mg elemental calcium + 400 IU vitamin D_3 QD	8 years (to be completed in 2007)
Das Gupta and Salti (planned study, 2006)	42	D_5	5–35 μg, QD	12 weeks

advanced breast (N = 25) or colorectal (N = 11) cancers. EB 1089 is a newly synthesized vitamin D analog that is much more potent in regulating cell growth and differentiation than cholecalciferol ($1\alpha,25(OH)_2D_3$), has a lower tendency to induce hypercalcemia, and can induce apoptosis in some types of cancer cells.[38] All patients received the EB 1089 solution for 5 consecutive days per protocol, and it was continued as compassionate treatment beyond that time in 21 cases for 10–234 days. The first 11 patients enrolled had also received a single dose one week before starting the schedule of protocol doses. The treatment doses used started at 0.15 μg/m^2 body surface area daily and were gradually increased to a maximum of 17.0 μg/m^2 daily.

All patients receiving the maximum dose suffered from hypercalcemic toxicity. This study identified the optimal dose of EB 1089 to be 7.0 μg/m^2 daily. Six of the patients receiving compassionate treatment for more than 90 days showed stabilization of their disease. EB 1089 was found to be much less calcemic than $1\alpha,25(OH)_2D_3$. Eleven patients in the protocol treatment phase experienced hypercalcemia, with 4 showing severe hypercalcemia at doses of 0.45, 12.4, and 17 μg/m^2. During the compassionate treatment phase, 10 patients experienced hypercalcemia, 6 of them severely. However, this study did not demonstrate any anti-tumor effect, as determined by an objective reduction in tumor volume, although six patients showed stabilization of their disease for over three months. Clinical trials evaluating the effectiveness of EB 1089 was then carried out in other cancer types as well.[38]

The Women's Health Initiative (WHI) Clinical Trial and Observational Study also included a vitamin D supplementation arm. Supplementation was primarily hypothesized to prevent hip and other fractures, and secondarily to prevent colorectal and breast cancer.[39] The WHI was established by the National Institutes of Health (NIH) in 1991 and the study involves over 161,000 postmenopausal women aged 50–79, who were enrolled in the study at 40 nationwide clinical centers between 1993 and 1998.

As indicated, one of the hypotheses being tested in the vitamin D arm of the WHI study is that women who receive calcium and vitamin D supplements will benefit with a lower risk of breast cancer than women receiving a placebo. This large-scale trial of a breast cancer chemopreventive agent is a 1:1 randomized double-blind trial using 1000 mg elemental calcium plus 400 international units (IU) of vitamin D_3 daily, versus a placebo. Participants take two pills per day. The planned completion date of the WHI study is 2007 and it is projected to enroll 45,000 women in the calcium and vitamin D supplementation arm. The complete findings of this study are eagerly awaited.

Results published in 2006 from the WHI study found that vitamin D and calcium were not protective against colon cancer in women.[40] A study finding such as this raises major questions regarding the cancer-vitamin D connection and requires reevaluation of the ongoing work in the area of vitamin D and cancer prevention and therapy. However, several factors in the study may have contributed to the finding that vitamin D had no significant beneficial effect on colon cancer.

First, at enrollment, the participants had mean total calcium (1151 mg) and vitamin D (367 IU) intakes that were twice the national average. Second, with about 40% of the study population not complying completely with the study medication regimen, there may have been insufficient numbers of study participants to demonstrate any beneficial effect on colorectal cancer. Third, the basic calcium and vitamin D doses used—1000 mg of elemental calcium and 400 IU of vitamin D_3—may have been insufficient to provide protection against cancer, as conceded by the authors. Fourth, the intervention period of seven years may have been too short to demonstrate an effect, given that colon cancer takes 10 to 20 years to appear.

Because the effects of vitamin D and calcium are target organ specific, a study not demonstrating a benefit from calcium and vitamin D supplementation on colorectal cancer does not definitively rule out the possibility that such supplementation may provide

protection against cancers in other organs. This study also only evaluated a single drug regimen for colorectal cancer and leaves open the possibility that other formulations and doses, targeted toward other cancers or even colorectal cancer, may have different results. For example, in experimental carcinogenesis models, vitamin D has no effect on lung cancer, whereas it suppresses development of breast and prostate cancer.

VITAMIN D_5

The first evaluation of D_5 as a chemopreventive agent for breast cancer will be conducted in our upcoming clinical trial. At the University of Illinois at Chicago (UIC) we have carefully designed a combined Phase I/II clinical trial to evaluate the safety and efficacy of $1\alpha(OH)D_5$ in patients with metastatic breast cancer. This safety/chemoprevention study, in addition to finding the maximum tolerated dose (MTD) for D_5, will monitor the clinical response as evaluated by decreases in measurable disease determined by physical examination, radiographic studies, and/or nuclear medicine scans.

The breast cancer trial with D_5 at UIC is a companion trial to another that will soon be conducted with D_5 and prostate cancer patients.[6] There are many similarities between breast and prostate cancer, which both respond to vitamin D. The latter trial will also be a phase I/II safety/chemoprevention study to determine whether $1\alpha(OH)D_5$ can safely delay prostate cancer recurrence when administered after definitive radiation therapy (RT).

In addition to epidemiologic and ecological studies, many animal studies have pointed to the possibility that vitamin D may be an effective chemopreventive agent against breast cancer. There are a number of good reviews on these topics.[41–44] These animal studies, which are the first steps in the process that a new chemopreventive agent must undergo, include preclinical studies in in vitro and in vivo animal experiments, followed by phase I, II, and III clinical trials for toxicity and efficacy.

CONCLUSION

Vitamin D and its analogs such as $1\alpha(OH)D_5$ may have a role to play in chemoprevention of breast cancer. Although a modest to moderate degree of toxicity may be acceptable in the context of chemoprevention to prevent disease recurrence, only minimal toxicity would be acceptable in the use of chemoprevention of primary breast cancer in healthy women. Thus, a strong focus on evaluating the known and potential side effect profiles of chemopreventive agents is urgently needed. Funding agencies involved in cancer research, such as the National Cancer Institute, must demonstrate strong leadership initiatives to identify and evaluate new chemopreventive agents and strategies to both deepen our understanding of cancer, and reduce its morbidity and mortality. Foundations and organizations involved in cancer education also play a strong role in increasing public and patient awareness of the importance of the research process in advancing the therapeutic armamentarium against cancer. Through well designed preclinical and clinical studies, we believe that efficacious chemopreventive agents can be identified and used to prevent primary and recurrent breast cancer.

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Differential expression of prohibitin is correlated with dual action of Vitamin D as a proliferative and antiproliferative hormone in breast epithelial cells

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Abstract

Our previous microarray analysis showed that *N*-methyl-*N*-nitrosourea (MNU) transformed MCF12F breast epithelial cells exhibited upregulation of several genes, including prohibitin, which was reversed by 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$) treatment. The *in silico* screening for putative transcription factor binding sites identified two VDR/RXR binding sites in the 1 kb promoter region of prohibitin. Other binding sites for EGR and GR which are also Vitamin D target genes were identified in this region, indicating that prohibitin is a potential target gene for Vitamin D. The combination of multiple binding sites also provides a basis for a possible dual regulation of prohibitin by Vitamin D. Prohibitin upregulation by $1\alpha(OH)D_5$ treatment at both transcription and translation level was observed in Vitamin D sensitive BT474 breast cancer cells, in which $1\alpha(OH)D_5$ significantly inhibited cell proliferation in normal culture condition. On the other hand, prohibitin down-regulation accompanied with Vitamin D mediated maintenance of proliferation of breast epithelial cells was observed under stressed condition. These results demonstrated that Vitamin D mediated antiproliferative activity in unstressed condition and growth maintaining activity under stressed condition involve differential expression of prohibitin.

Keywords: Prohibitin; Vitamin D receptor; Breast cancer cells; CYP24 promoter; Proliferation

1. Introduction

We have previously shown that a relatively noncalcemic analog of Vitamin D, 1α-hydroxy-24-ethyl-cholecalciferol ($1\alpha(OH)D_5$) is a promising chemopreventive agent in experimental mammary carcinogenesis models [1]. Using microarray analysis we identified several genes that are differentially regulated in carcinogen transformed human breast epithelial cells as compared to the untransformed cells (MCF12F) and the expression of a few genes including prohibitin, TCTP1 and thioredoxin was reversed with $1\alpha(OH)D_5$ treatment in the transformed cells [1]. Since prohibitin has been reported to have interactions with the cell cycle checkpoint molecules including E2F [2], p53 [3] and pRB [2] as well as shown to exhibit tumor suppressive and antiproliferative effects, we selected prohibitin to determine if it has a functional significance in Vitamin D action. Prohibitin is a highly conserved protein and is localized into the inner membrane of the mitochondria, where it has a role as a mitochondrial chaperon protein, as a complex with BAP37 in the maintenance of mitochondrial function [4]. Moreover, it is also reported to be localized in the nuclei of breast cancer cells and mediating hormone response in prostate cancer and ovary granulosa cells [2]. Recent reports indicate that prohibitin is up-regulated in tumor cells as compared to normal cells [5]. Our attempts to characterize the role of prohibitin in VDR function led to identification of prohibitin as a target gene of Vitamin D, which is dually regulated by Vitamin D and involved in Vitamin D mediated cellular responses.

2. Materials and methods

2.1. Cell culture

BT474, MCF-7, human breast cancer cells, were cultured in MEM containing 10% FBS as described previously [6].

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MCF12F cells were cultured in DMEM/F12 containing 5% horse serum, supplemented with 20 ng/ml EGF (Invitrogen), 10 μg/ml insulin (Sigma), 100 ng/ml cholera toxin (Sigma) and 0.5 μg/ml hydrocortisone. To induce stress in MCF12F cells, culture medium was replaced with MEM containing 2% FBS. The Vitamin D analog, $1\alpha(OH)D_5$ was synthesized and dissolved as described previously [1]. $1\alpha,25(OH)_2D_3$ was purchased from Sigma.

2.2. Cell proliferation assay

Cell proliferation was evaluated using MTT assay [6] and BrdU (bromodexyuridine) incorporation assay. For BrdU incorporation assay, 10 μM BrdU (Calbiochem, La Jolla, CA) was added to the medium at 2 h before harvesting cells. Cells were trypsinized, washed with PBS and fixed in 1% paraformaldehyde in PBS for 15 min, followed by incubation in PBS containing 0.2% Tween-20 for 30 min at 37 °C. Cells were then incubated with mouse monoclonal anti-BrdU antibody (Calbiochem) overnight at 4 °C, then washed twice, incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After three washes with PBS, cells were subjected to FACS analysis, a total of 20,000 events were measured per sample.

2.3. RNA interference

10^5 cells per well were seeded in six well plates in culture media containing 5% FBS and incubated overnight. SiRNA against PHB (siPHB) and non-silencing siRNA (siCon, Qiagen, cat. no. 1022076) were transfected using the RNAiCarrier kit (Epoch Biolabs, Sugar Land, TX). Cells were first incubated with siRNA complex (SiRNA at 80 nM, SiRNA:RNAi Carrier = 1:6) for 6 h in Opti-MEM (Invitrogen) containing 2% FBS, then FBS was increased to 5%. After overnight incubation, cells were incubated in fresh culture medium containing 5% FBS for 2 days in the presence or absence of $1\alpha,25(OH)_2D_3$, cells were then subjected to BrdU incorporation assay. Prohibitin knock-down was verified at the same time using western blot [6]. siPHB oligonucleotide duplex (SiPHB sense: 5′-CCCAGAAAUCACUG-UGAAAdTdT-3′, antisense: 5′-UUUCACAGUGAUU-UCUGGG-dTdT-3′) were designed

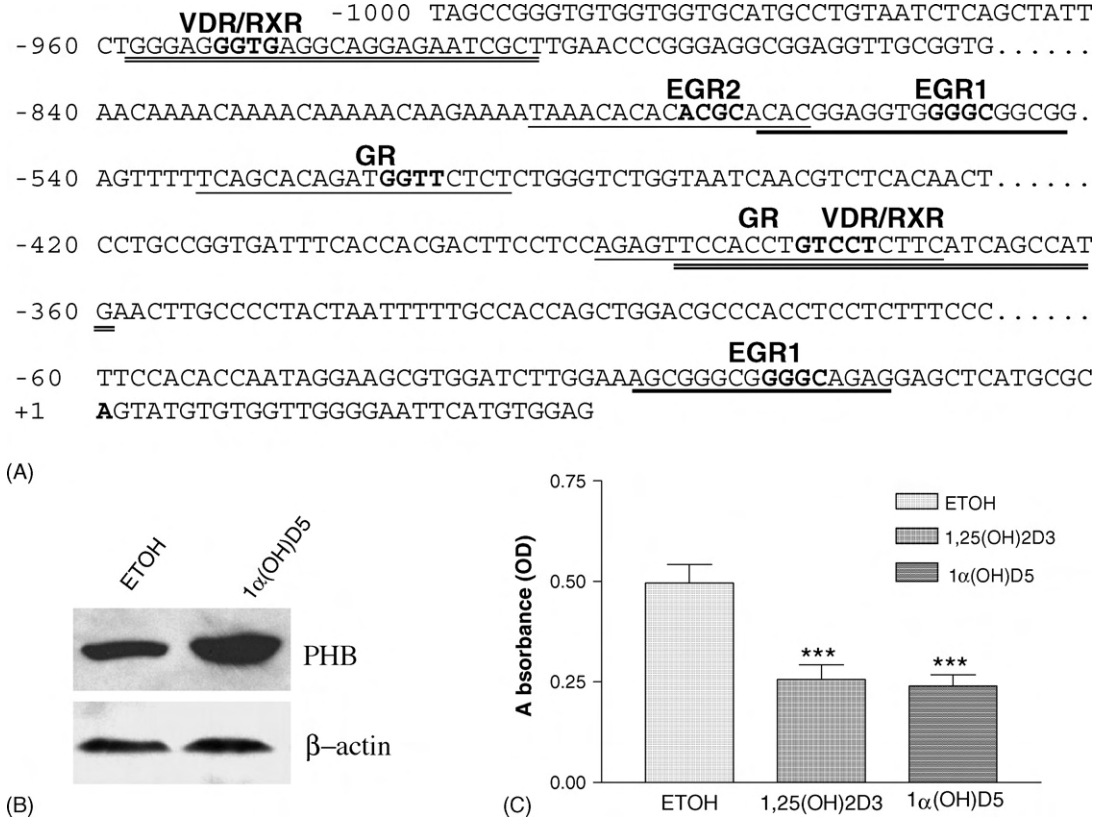

Fig. 1. Prohibitin is a target gene of Vitamin D. (A), *in silico* analysis of potential VDR/RXR binding sites in the promoter region of prohibitin gene. The core sequence (in bold face) of the putative transcription binding sites for VDR/RXR, EGR and GR with high identity to the authentic core and matrix sequences as identified by MatInspector V2.2 and promo are underlined. Nucleotides are numbered negatively to the left of the sequence with nucleotide +1 corresponding to the transcription start site. (B) and (C) Upregulation of prohibitin by Vitamin D correlates with its inhibition of cell proliferation in BT474 cells. (B), Western blot analysis of cell lysates from BT474 cells treated with 0.5 μM $1\alpha(OH)D_5$ for 24 h. $1\alpha(OH)D_5$ treatment significantly increased prohibitin expression. β-Actin was used as an internal control. (C), MTT assay of BT474 cell proliferation after 4 day treatment with $1\alpha(OH)D_5$ (0.5 μM) and $1,25(OH)_2D_3$ (100 nM). Results are expressed as mean ± S.E.M. of three independent experiments with eight wells per treatment in each experiment. $^{***}p < 0.001$ compared to control.

to target the 3′UTR region at base pair 974-993 of sequence NM_002634 and synthesized by IDT Inc (Coralville, IA). Negative control siRNA (non-silencing RNA, sense: 5′-UUCUCCGAACGUGUCA-CGUdTdT-3′, antisense: 5′-ACGUGACACGUUCGGAGAAdTdT-3′) has no homology to known mammalian genes.

3. Results

3.1. Prohibitin upregulation by 1α(OH)D_5 correlated to its inhibition of proliferation in BT474 cells

Our earlier studies have shown that prohibitin could be involved in cellular response to 1α(OH)D_5 in breast epithelial cells [1]. To further address this issue, we analyzed the promoter region of prohibitin to examine if potential VDR binding sites exist. *In silico* analysis identified two potential DNA binding sites for VDR/RXR in the 1 kb promoter region and multiple EGR and GR binding sites (Fig. 1A). Both EGR and GR are Vitamin D target genes [7,8, unpublished data]. A time-course analysis of prohibitin mRNA expression in BT474 cells demonstrated that prohibitin mRNA level increased by ∼two-fold after 8 h treatment with 1α(OH)D_5 (0.5 μM) (data not shown). Prohibitin protein upregulation by 24 h incubation with 1α(OH)D_5 was confirmed by western blot in the same cell line (Fig. 1B). Cell proliferation assay demonstrated that 1α(OH)D_5 inhibited cell proliferation by 50% after 4 day treatment in BT474 cells in normal culture condition (Fig. 1C). These data identified prohibitin as a Vitamin D target gene and that prohibitin upregulation by1α(OH)D_5 correlates to its inhibition of cell proliferation in BT474 cells.

3.2. Prohibitin downregulation by 1α,25(OH)$_2D_3$ and RNAi in stressed MCF-7 cells was accompanied by increased BrdU incorporation

Given that prohibitin is a Vitamin D target gene and can potentially contribute to the antiproliferative action of Vitamin D, we investigated the effect of knock-down of prohibitin by RNAi on cell proliferation in MCF-7 cells. Transfection of siPHB for 24 h, followed by 48 h incubation with regular culture medium containing 5% FBS decreased prohibitin protein by more than 70% as evaluated by western blot analysis (Fig. 2C). BrdU incorporation assay using FACS analysis demonstrated a significant increase (>three-folds) in BrdU-labeled cells after prohibitin knock-down (Fig. 2B) in comparison to non-silencing control siRNA (siCon) transfected cell (Fig. 2A). Surprisingly, contrary to our expectation, 1α,25(OH)$_2D_3$ treatment in siCon transfected MCF-7 cells significantly decreased prohibitin level

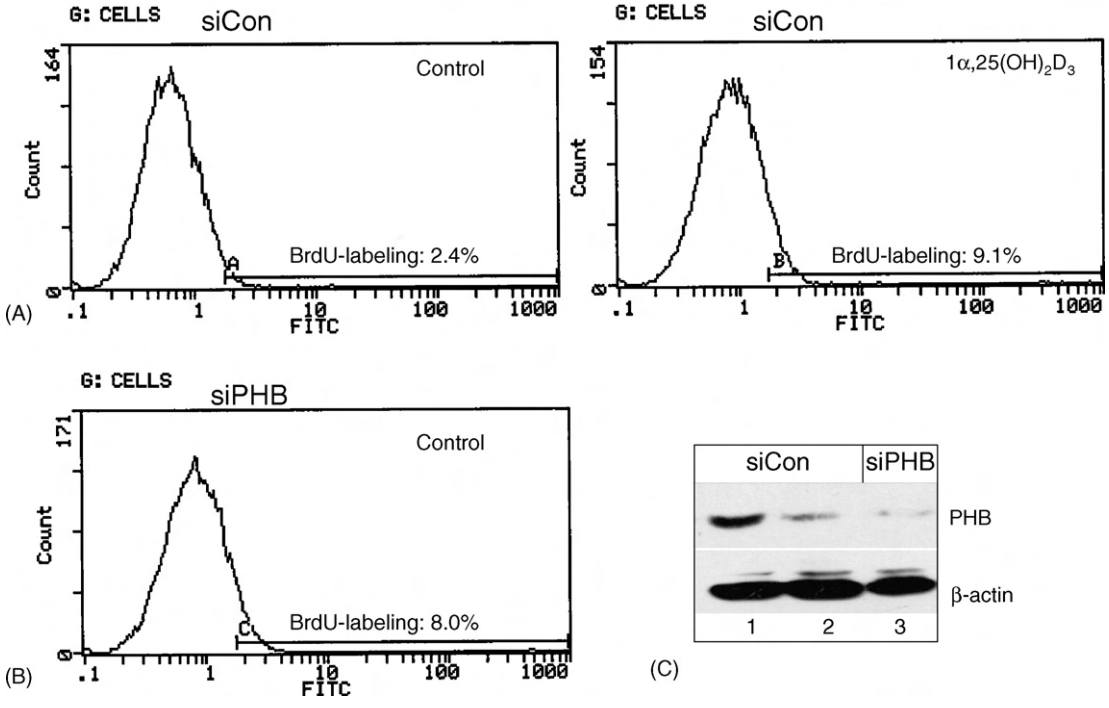

Fig. 2. Downregulation of prohibitin by 1α,25(OH)$_2D_3$ or siRNA in stressed MCF-7 cells correlates with enhanced proliferative activity. (A), cells were transfected with siCon and incubated in the medium containing low serum to cause stress and then treated with 100 nM 1α,25(OH)$_2D_3$ for 48 h. (B), transfection of siPHB served as a control for prohibitin downregualtion. Both 1α,25(OH)$_2D_3$ and siPHB transfection downregualted prohibitin under the experimental condition, which was accompanied by increased BrdU incorporation in MCF-7 cells. (A), representative FACS analysis of BrdU incorporation in siCon transfected MCF-7 cells with ETOH (lfet) or 1α,25(OH)$_2D_3$ (right) treatment. (B), BrdU incorporation analysis of siPHB transfected MCF-7. (C), Western blot analysis of prohibitin expression corresponding to above treatments; lane 1 siCon transfection with ETOH treatment; lane 2, siCon transfection with 1α,25(OH)$_2D_3$ treatment; lane 3, siPHB transfection without treatment.

(Fig. 2C, lane 2) and increased cell proliferation (Fig. 2A), which was comparable to the effects of prohibitin knockdown by siRNA.

3.3. Dual effects of 1α,25(OH)$_2$D$_3$ in MCF12F cells under normal and stressed condition correlate to prohibitin regulation

Considering the results from siRNA transfected cells and the stress caused by transfection itself and transfection condition (low serum), we hypothesized that prohibitin could be a stress-associated protein, while Vitamin D could play growth supportive role in the stressed cells by inhibiting prohibitin expression. We used MCF12F cells to further address this issue. Since MCF12F cells are generally grown in nutrient medium supplemented with growth factor and hormones, replacement of the nutrient medium with regular medium containing low serum induces significant stress to the cells. As shown in Fig. 3A, in normal culture condition, 4 day treatment with 100 nM 1α,25(OH)$_2$D$_3$ significantly inhibited cell proliferation by ∼40%; while in stressed culture condition, 1α,25(OH)$_2$D$_3$ stimulated cell proliferation by ∼two-fold. The dual effect of 1α,25(OH)$_2$D$_3$ was also reflected in prohibitin regulation (Fig. 3B). In normal culture condition, 48 h treatment with 1α,25(OH)$_2$D$_3$ upregulated prohibitin (after normalized to β-actin), while in stressed condition, 1α,25(OH)$_2$D$_3$ inhibited prohibitin expression. The dual regulation of prohibitin by 1α,25(OH)$_2$D$_3$ in different culture condition correlated to its dual effects on cell proliferation as diagramed in Fig. 3C.

4. Discussion

In the literature Vitamin D is often reported to inhibit both proliferation and apoptosis in cancer cells [9,10]. However the detailed signaling pathway for such dual effects has been poorly understood. In this study, we identified prohibitin as a Vitamin D target gene, which is differentially expressed in cellular responses to Vitamin D in normal and stressed conditions.

Prohibitin is a highly conserved protein and localizes to many cellular compartments and might have distinct but overlapping function in each of these [2]. Its role as a tumor-suppressor protein, however, is still controversial [2]. Our data show that prohibitin is dually regulated by Vitamin D in breast epithelial cells depending on the culture condition. *In silico* analysis identified two potential VDR binding sites

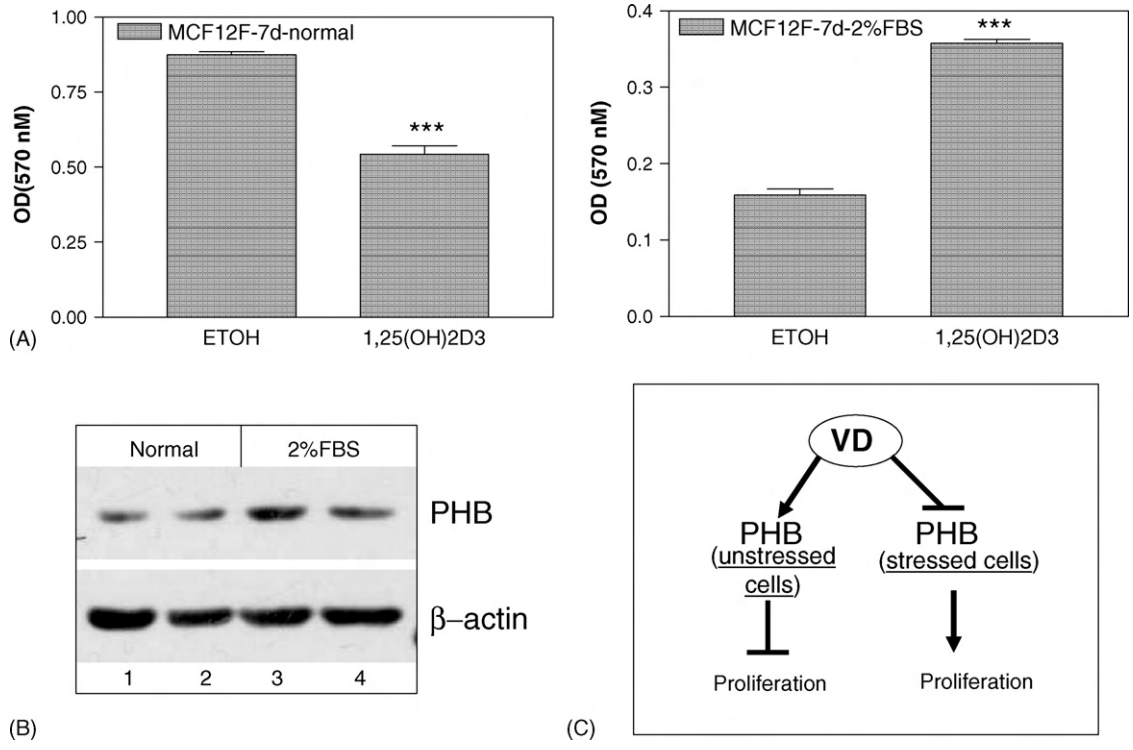

Fig. 3. Prohibitin downregulation by 1α,25(OH)$_2$D$_3$ in stressed MCF12F cells correlates to enhanced proliferative activity. (A), dual regulation of cell proliferation by 1α,25(OH)$_2$D$_3$ (100 nM) in normal culture condition (left) and stressed condition (low serum without supplements) (right). Cells were treated for 4 days and subjected to MTT assay. Results are expressed as mean ± S.E.M., $^{***}p < 0.001$ compared to control. (B), prohibitin regulation by 1α,25(OH)$_2$D$_3$ in normal culture condition and stressed condition (2%FBS). MCF12F cells were treated for 48 h and subjected to western blot analysis; lane 1 and 3, ETOH (control); lanes 2 and 4, 1α,25(OH)$_2$D$_3$. (C), Schematic model of dual regulation of prohibitin by active Vitamin D analog (VD) in relation to its proliferation-regulatory effects. Under stressed condition, active Vitamin D plays a protective role by downregulating prohibitin and maintaining cell proliferation; while in normal culture condition, Vitamin D upregulates or fine-tunes prohibitin which is involved in its antiproliferative effect.

and multiple putative EGR and GR binding sites in the promoter region of prohibitin. EGR and GR are also involved in cell stress [11,12]. The combination of these binding sites in promoter region provides a basis for dual regulation of prohibitin by Vitamin D. Initially, the siRNA experiments were designed to evaluate the function of prohibitin in relation to cell proliferation and not stress. However the downregulation of prohibitin by 1α,25(OH)$_2$D$_3$ in siCon tranfected MCF-7 cells suggested that prohibitin may be a molecule associated with stress. Chemical reagents used for transfection are generally toxic, causing significant stress to cells. In addition, transfection is generally introduced in serum-starved medium, which also induces stress to cells. Our studies with MCF-12F cells using different culture condition confirmed this hypothesis and provided evidence for both up- and downregulation of prohibitin by active Vitamin D. Consistent with our finding, prohibitin was previously identified as a protein responding to mitochondrial stress [4]. In addition prohibitin was shown to actively translocate from nuclei to cytoplasm when cells were stressed with camptothecin, a strong inducer of apoptosis [3].

There is overwhelming evidence demonstrating protective function of Vitamin D in addition to its antiproliferative effects. Zhang et al. [9] previously showed a dual effect of 1,25(OH)$_2$D$_3$ in ovarian cancer cells. Pretreatment of these cells with 1α,25(OH)$_2$D$_3$ decreased apoptosis induced by TRAIL and Fas ligand, however the persistent 1α,25(OH)$_2$D$_3$ treatment induced apoptosis in ovarian cancer. Similarly, Riachy et al. [10] reported that 1α,25(OH)$_2$D$_3$ protects human pancreatic islets against cytokine-induced apoptosis via downregulation of the fas receptor. Considering that many genes involved in stress such as EGR, GR and prohibitin are Vitamin D target genes, Vitamin D seems to have protective anti-stress function. Cells can undergo transformation under stress; with Vitamin D protection, the stress-associated transformation can be significantly inhibited. This supports the notion for Vitamin D as a possible chemopreventive agent.

In summary, the results presented here provide evidence for the possible mechanism by which Vitamin D can function both in maintaining cell proliferation while the cells are undergoing stress as well as an antiproliferative hormone in breast epithelial cells. We show here for the first time that prohibitin is both a Vitamin D target gene and a stress-associated molecule and is involved in Vitamin D mediated cellular functions in breast epithelial cells.

Acknowledgement

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ORIGINAL ARTICLE

Designing a Randomized Phase I/II Prostate Cancer Chemoprevention Trial Using 1α-Hydroxy-24-Ethyl-Cholecalciferol, an Analogue of Vitamin D_3

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ABSTRACT

Prostate cancer continues to be a significant source of morbidity and mortality among older men. One possible means of reducing its impact on overall health and vitality is via cancer chemoprevention, both in the population that is unaffected but at some risk and in those who have undergone some form of curative therapy after the onset of the disease. Chemoprevention holds significant promise, but large phase III clinical trials evaluating chemopreventive agents in prostate cancer can require vast numbers of enrollees and require the commitment of significant financial resources and time before any therapeutic benefit may become apparent. One technique to shorten the time required for chemoprevention clinical trials is to use surrogate endpoint biomarkers in place of the currently used actual endpoints of cancer incidence or survival. The validation of such surrogate endpoint biomarkers will require small, well-designed phase I and/or II trials to accumulate data on the modulation of the surrogate biomarkers and the endpoints of cancer incidence or survival by the chemopreventive agent. Careful statistical correlation and clinical validation of the data will then allow us to justify the use such surrogates in place of the actual endpoint in large, randomized trials, potentially shortening trial duration, improving financial efficiency, and accelerating approval of the chemopreventive agent. To that end, we first review the theoretical construct of cancer chemoprevention trials with particular reference to prostate cancer. We thereafter describe the design of a small, randomized, double-blinded, placebo-controlled phase I/II clinical trial of an analogue of vitamin D, vitamin D_5, which we believe could serve as a model for data accumulation on surrogate biomarkers and correlation with other clinical endpoints. *(Cancer J 2004;10:357–367)*

KEY WORDS

Chemoprevention, vitamin D, prostate cancer, radiation therapy, randomized clinical trial, phase I/II

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Prostate cancer, the risk factors for which include older age, family history, ethnicity, and race,[1] is one of the more common cancers afflicting men in the United States and Western Europe. One autopsy study, for instance, documented prostatic carcinomas in as many as 29% of men between the ages of 30 and 39 years and in 63% of those between the ages of 60 and 69 years.[2] Because of the often decades-long latency period for progression from normal tissue to prostate cancer, it is believed that effective chemoprevention could be a viable means of reducing the incidence of prostate cancer. To that end, large, randomized, double-blinded, phase III chemoprevention clinical trials, such as the Prostate Cancer Prevention Trial (PCPT)[3-5] and the Selenium and Vitamin E Cancer Prevention Trial (SELECT),[6,7] were initiated. However, the major endpoint in these large studies is the onset of prostate cancer, which, despite its significant public health impact, has only a low annual incidence (0.27% in men ≥ 34.4 years of age).[8] This, coupled with prostate cancer's long latency period, may necessitate prolonged trial monitoring before any reduction of prostate cancer incidence is demonstrated. Improvements in trial design and efficiency are thus eagerly

awaited, not merely in prostate cancer studies but in other types of cancer studies as well.

Short and efficient phase III trials could be theoretically accomplished with fewer resources and patients if validated surrogate endpoint biomarkers (SEBs) that were accepted by the US Food and Drug Administration (FDA) were available in addition to the currently accepted endpoint of actual disease presence. It is also critical that SEBs be initially examined clinically in small phase I and II trials before they are used and further validated in phase III trials.[9] Thus, in the case of prostate cancer, investigators would test the efficacy of current or new chemopreventive drugs and at the same time gather valuable data on potential SEBs, such as prostate specific antigen (PSA) modulation, prostatic intraepithelial neoplasia (PIN) progression, and molecular/genetic biomarkers in small, randomized, phase I/II studies. Hence, the SEBs' statistical and clinical correlation with, and their predictive potential for, the endpoint of prostate cancer per se can be more strongly established. Moreover, important design elements, such as population selection, randomization, quality and type of SEBs, and quality of life (QOL) parameters can be carefully integrated and optimized for the type of cancer and the drug being investigated.[8] Such multipronged initial efforts could potentially lead to shorter and more efficient phase III trials.[10]

The active form of vitamin D, 1α,25-dihydroxycholecalciferol [1α,25$(OH)_2D_3$ or vitamin D_3], or calcitriol, because of its antiproliferative and differentiation-inducing properties, has been extensively investigated as a potential chemopreventive agent. Some of these studies have detected promise in cutaneous, colorectal, breast, and prostate cancer chemoprevention.[11-14] Because of the significant toxicity of vitamin D_3—secondary to hypercalcemia induction at pharmacologic doses—analogues less likely to induce hypercalcemia have been designed and developed for use in cancer chemoprevention. These analogues have anti-proliferative potential at least equal to if not greater than that shown by vitamin D_3.[15] One such analogue that has undergone significant preliminary testing is 1α-hydroxy-24-ethyl-cholecalciferol [1α$(OH)D_5$ or vitamin D_5]. Reportedly, vitamin D_5 is the least toxic of the vitamin D series of compounds D_2 through D_6 and has been examined in several preclinical studies.[16]

In this paper, we briefly review the concepts underlying chemoprevention, clinical trials, and surrogate endpoints before detailing our experience in designing a combined phase I/II randomized clinical trial to evaluate the effectiveness of 1α-hydroxy-24-ethyl-cholecalciferol [1α$(OH)D_5$] in prostate cancer chemoprevention. Our short overview of each of these is limited primarily to the role played by each in prostate cancer.

CHEMOPREVENTION

Since its introduction in 1976,[17] chemoprevention has been accepted as an essential ally in cancer therapy.[18,19] Generally, cancer chemoprevention agents function via three broad mechanisms: their carcinogen-blocking, antioxidant, and antiproliferative/antiprogression activities.[20] Depending on the type of cancer being targeted, chemopreventive agents can be dietary factors, nutritional supplements, hormones, intra- or extracellular receptor modulators, growth factor inhibitors, anti-inflammatory agents, and specifically directed gene therapy, among others. In its broadest sense then, cancer chemoprevention attempts to use natural, synthetic, biologic, or chemical agents to suppress, reverse, or prevent carcinogenic progression to invasive cancer.[21]

Although simple in concept, chemoprevention harbors significant promise in cancer control because it inhibits the formation of the precancerous state and impedes or halts carcinogenic progression. Chemoprevention clinical trials have been initiated or have been considered for virtually all cancers, including bladder cancer,[22,23] prostate cancer,[24,25] gastric cancer,[26,27] hepatocellular carcinoma,[28,29] breast cancer,[30,31] head and neck cancer,[32,33] colorectal cancer,[34-36] and lung cancer.[37,38] Thus, one could conceivably argue that chemoprevention will become an increasingly important tool in our therapeutic armamentarium against all types of cancer, especially those with long latency periods from mutagenesis to cancer.

Although chemoprevention itself may be a novel, yet simple, concept, the epidemiologic, experimental, and/or other preclinical studies considered necessary to provide evidence that a particular drug or intervention can be beneficial in the prevention of a particular type of cancer are complex. Moreover, once a beneficial effect has been established by, or at least inferred from, these studies, one then faces the difficult task of designing appropriate clinical trials to test the interventions.

In the next section, we very briefly review some of the concepts underlying clinical trials before delving into the relationship between actual endpoint biomarkers and SEBs.

CLINICAL TRIALS

Clinical trials represent today's frontiers of medicine. Each properly conducted and completed trial, regardless of outcome, advances our understanding of disease processes and patient treatment options in a setting that is clinically as safe and as devoid of bias as possible. Clinical trials are routinely classified as being phase I, II, or III. A phase I trial is very often unrandomized, enrolls a small number of patients, and focuses primarily on

patient safety, drug doses, pharmacokinetics, and pharmacodynamics, as well as a very limited estimation of patient response to the treatment. The phase II trials, which are sometimes randomized, estimate treatment efficacy at a more limited range of doses, while continuing data collection on adverse events. Thus, a phase II trial, with only a modestly larger number of patients, focuses more closely on the clinical benefit to be derived from the drug or the intervention. Such studies also provide essential guidance regarding the degree of clinical and statistical response, if any, that one could anticipate from the drug in a phase III trial.[9] Moreover, if an intervention has been documented in preclinical studies to have only nominal side effects, phases I and II could potentially be combined into a single trial. In the phase III trials, however, large numbers of subjects are sought and then randomly assigned to various treatment and control arms to estimate the benefits of the intervention with the expectation that findings may be generalized and applied to the population from which the trial participants were derived.[39]

In cancer, clinical trials are routinely organized to evaluate a therapeutic or a chemoprevention strategy. Ideally, chemoprevention trials would target individuals who are currently healthy or who are healthy but have a significantly higher than normal risk of developing cancer in the future. The underlying benefit to such a patient population lies in the anticipated reduction in the incidence of the cancers being targeted. Examples of such targeted patient populations include

- Patients with head and neck precancerous states, such as leukoplakia for head and neck squamous cell cancers[33]
- Smokers for lung cancer[40]
- Heavily sun-exposed individuals for various skin cancers[41]
- Individuals with colonic adenomas for colonic cancer[42,43]
- Prostate biopsy-negative men with varying degrees of PIN but only modestly elevated serum PSA levels[4,5]

In addition, some chemoprevention trials are opened to cancer patients who have undergone or will soon undergo some form of therapy that is considered curative, such as surgery or radiation therapy (RT). In such patients, the trials test the hypothesis that the planned chemoprevention will supplement or even augment the curative therapy by reducing or eliminating the likelihood of recurrence or of a second primary tumor. Such tertiary patient populations have included those with head and neck squamous cell cancers,[44] breast cancer,[45] lung cancer,[46] colorectal cancer,[47] or prostate cancer.[48]

Hence, depending on patient selection, chemoprevention therapies can be targeted toward primary, secondary, or tertiary prevention. In primary prevention, it can occasionally be difficult to recruit healthy individuals into chemoprevention trials and maintain their compliance with the treatment regimen because their self-perception of personal risk may be low. In addition, any intervention or chemopreventive drug in this population must have minimal side effects. Sometimes, too, phase I chemoprevention studies are performed in the tertiary prevention population because the safety of the test drug may not yet have been adequately established for use in the healthy or healthy but still higher-risk population. In patients already diagnosed with cancer, a greater degree of uncertainty about the drug's toxicity may be considered acceptable, given its potential benefit.

However, therapeutic cancer trials are exclusively directed toward patients who have a diagnosis of cancer and are awaiting therapy. Such trials often compare the efficacy of different treatments or examine the superiority of one type of treatment over another. On occasion, they may investigate the use of an experimental drug or therapy on a seemingly incurable form or stage of cancer. The endpoints in these studies can include QOL improvements, length of disease-free survival, extent of local or systemic control of disease, or outright cure. Patients in therapeutic trials are often seriously or even terminally ill. Yet, because these early investigational or untried interventions may represent the only clinical option available to palliate symptoms, prolong life, induce disease remission, or cure the disease, a higher degree of drug toxicity would be considered an acceptable risk, given the potential benefit. Similar criteria also underlie the bases of patient selection in the therapeutic trials of other medical specialties.[49]

The key element of a clinical trial then, apart from its targeted patient population and the interventions planned, is the disease endpoint it is designed to monitor. In the subsequent section, we review briefly the basic principles of endpoints and SEBs before proceeding to discussion of the vitamin D_5 clinical trial.

ENDPOINTS AND SURROGATE ENDPOINT BIOMARKERS

The definitive endpoints of any disease are final clinical outcomes that are relevant to the patient and/or the health community. These may include death, loss of function of an organ, a diagnosis of cancer, and a cardiac event. SEBs are alternatives to the actual endpoint, the modulations in which correlate with and predict, statistically and clinically, the true endpoint. Such SEBs are as a rule attained faster, require less invasive monitoring, and are less costly to observe than the true end-

point.[50-52] Some selected SEBs for neoplastic and nonneoplastic diseases include

- Bronchial metaplasia for lung cancer[53]
- Plasma cholesterol levels for the endpoint of a cardiac event[54]
- Cervical intraepithelial neoplasia for cervical cancer[55]
- CD-4 cell counts and plasma viral loads for death or opportunistic infections in human immunodeficiency virus–infected patients[56]
- Changes in colonic adenoma number/histology for the likelihood of colonic cancer[8]
- Intraocular pressure for vision loss in glaucoma[57]

In chemoprevention trials, SEBs are particularly useful for estimating the effects of preventive interventions on the endpoint of cancer incidence. Especially in phase II chemoprevention trials, appropriate SEBs may permit rapid preliminary assessment of efficacy, dose response, and suitability for progression to phase III trials. In the case of prostate cancer, potential SEBs include serum PSA level, PSA doubling time, serum alkaline phosphatase level, histochemical/molecular monitoring of apoptotic biomarkers, changes in degree or new occurrence of PIN, cell/nuclear morphometry, chromosomal changes, and QOL parameters. Any modulations noted in SEB measures must actually predict increased/decreased prostate cancer risk, and these must be appropriately validated before the chemopreventive efficacy is accepted and used. This validation necessitates the fulfillment of four criteria by the SEB:[9]

1. The SEB is differentially expressed between normal and tumor tissue.
2. The SEB can be modulated by the planned intervention.
3. The SEB modulation by the intervention can be correlated with clinical response.
4. The SEB modulation by the intervention correlates with long-term cancer development.

Validation of SEBs for use in clinical trials is statistically a complex and demanding task whose methodology has been detailed elsewhere.[58,59] However, brief mention is made here of SEB validation with respect to prostate cancer chemoprevention.

The optimal SEB for any cancer will lie in the pathway leading to the endpoint and will directly affect the incidence of the endpoint. The ideal means of establishing the validity of a SEB as a substitute for the actual endpoint is by conducting a clinical trial with the endpoint that the SEB is designed to replace.[51] However, validating such a benchmark is impractical because cancer can take decades to develop. Hence, initial statistical correlation will more than likely be extracted from other in vitro, in vivo, or epidemiologic studies in which the SEB was also monitored in addition to the actual endpoint. Before an SEB is selected for further study, an estimate of cancers that can be attributed to the SEB must be made. This "attributable proportion" or AP is represented by the formula:

$$AP = S\,(1 - 1/R),$$

where R = relative risk and S = sensitivity. A value close to 1.0 suggests that the SEB under evaluation is very likely to lie in the pathway leading to the cancer endpoint. In contrast, values ≤ 0.5 for the AP would suggest that 50% or less of the cancers can be attributed to the SEB.[9]

In addition, under the null hypothesis, the SEB must yield the same result as the true endpoint. This fundamental criterion and others regarding the statistical principles for the use of SEBs were initially articulated by Prentice.[60] Those statistical beginnings have been gradually refined as the sophistication of statistical methodology improved.[52,61,62] One refinement, for example, is the concept that any changes in SEB must actually meet the requirement of "predicting" the likelihood of the actual endpoint rather than merely "correlating with" it.[61] Thus, before any conclusions of therapeutic efficacy can be drawn from SEB modulation by an intervention, such modulations of the SEB must also concordantly predict the effect on the actual endpoint. To rephrase this as an example familiar to prostate cancer, any intervention that reverses or decreases the rate of transformation to high-grade PIN should also actually translate into a decrease in prostate cancer incidence.

Even when SEB modulation by an intervention appropriately predicts the endpoint in preliminary studies, its validity can be called into question after large, randomized trials produce contradictory outcomes, such as hormone replacement therapy and cardiac disease in postmenopausal women.[63] Hence, even meticulous prephase III trial substantiation of an SEB cannot guarantee that it will perform in a similar manner in the randomized drug/placebo treatment protocol of an actual phase III trial.[61] Thus, SEBs to be used in phase III trials must be carefully selected. Even if their use is meticulously validated before they are selected, any reliance on them must be made with the stipulation that they can be quickly superseded by newly accumulating evidence.

Having briefly reviewed the concepts of chemoprevention, clinical trials, and SEBs, we now describe our experience in the design of a randomized phase I/II clinical trial to test the efficacy of a vitamin D analogue in patients with prostate cancer.

DESIGNING A POSTRADIATION THERAPY CHEMOPREVENTION TRIAL USING VITAMIN D_5

Rationale

RT and radical prostatectomy (RP) are the two major treatments for nonmetastatic prostate cancer, with essentially no difference in long-term patient outcomes.[64] At diagnosis, approximately 30%–50% of patients with nonmetastatic prostate cancer elect to undergo RT instead of RP. Of these, ~ 30%–40% at some point face biochemical and/or clinical failure despite this treatment option. Such failure is associated with poor prognostic factors on initial presentation. These prognostic factors, which include patient ethnicity, American Joint Committee on Cancer disease stage, pretreatment PSA level, pre-RT PSA nadir, and Gleason score, are each independent predictors of PSA relapse-free survival.[65-67]

Patients who do not respond to RT very likely do so because of clonal growth of radioresistant cancer cells or because of malignant clones arising from precancerous cells. In RT, because the prostate gland is permitted to remain in situ, the intrinsic "stimuli" that initiated mutagenesis and allowed progression to the original cancer can continue to exert their influence on the prostatic cells. Thus, the potential for recurrence is present for the remainder of the patient's life. After diagnosis of recurrence or of biochemical failure, these patients may face the grim prospect of undergoing continuous or intermittent androgen blockade, with all its associated side effects, including hot flashes, loss of libido, erectile dysfunction, tiredness, gynecomastia, and loss of bone mineral density, essentially for the rest of their lives. Very rarely, such a patient may choose to undergo salvage RP instead, if that option is offered. However, its benefits are uncertain, its complication rate significant, and its long-term outcome unknown. Hence, it would be extremely beneficial to the patients with prostate cancer who have undergone RT (perhaps even those who have undergone RP) if a chemopreventive agent that could delay or prevent the onset of biochemical failure or cancer recurrence were available.

Vitamin D_3, or calcitriol, has antiproliferative and differentiation-inducing properties that make it a potential chemopreventive agent for multiple cancers, including prostate cancer. Because of its hypercalcemic toxicity at pharmacologic doses, however, its less toxic analogues are now appearing to be better suited for a role in chemoprevention. One such analogue is vitamin D_5, or 1α-hydroxy-24-ethyl-cholecalciferol [1α(OH)D_5]. This compound, designed by Mehta and colleagues,[68] and manufactured under FDA "good manufacturing practice" guidelines, has been slated for use in an upcoming breast cancer phase I trial. Preclinical toxicity studies have also been completed in at least two separate species as required by the FDA.

Design Considerations

The major design considerations for chemoprevention trials in humans include identifying a chemopreventive agent, defining the type of clinical study (phase I, II, or III) and its duration, selecting a target population, selecting biomarkers for toxicity monitoring, choosing appropriate SEBs for disease monitoring, and using statistical modeling.[8,39,69] Within each category, however, design criteria must incorporate patient safety guarantees, appropriate statistical principles, and sufficient flexibility to modify drug/intervention regimens and to respond to institutional review board concerns.

Before initiating the design phase for our study, we carefully reviewed the design details underlying two recent large-scale, randomized phase III trials (PCPT and SELECT). After this review, we incorporated the features that, in our estimation, would optimize our design and maximize the potential for a clinically and statistically valid outcome of this randomized phase I/II study.[70]

The Prostate Cancer Prevention Trial (PCPT) is a large, randomized, double-blinded, placebo-controlled, period prevalence, and point prevalence study aimed to determine the usefulness of finasteride in reducing the incidence of prostate cancer. Begun in 1994, its design incorporated a three-month enrollment period during which all participants received the placebo before they were randomly assigned into treatment and control arms. In addition to any diagnostic biopsies performed during the 7-year treatment phase, all participants surviving to the end-of-study were expected to undergo a prostate biopsy. Its "period prevalence" design for the endpoint of prostate cancer incidence was decided on after much discussion among the study investigators. This allowed calculation of overall prostate cancer incidence during the 7 years of the trial, as well as "point prevalence" of prostate cancer at the 7th-year biopsy. Study participants were males > 55 years of age with normal PSA levels (≤ 3 ng/mL) and no other significant comorbid disease.[3]

This study was halted ~ 15 months before scheduled completion when its monitoring committee determined that the robust statistical differences between the treatment and the placebo groups were unlikely to improve in the time remaining. In the recently published summary of the trial findings, it was observed that finasteride decreased the period prevalence of prostate cancer by 24.8% over the 7-year period. However, the unexpected finding that a significantly higher percentage of prostate tumors discovered in the finasteride-treated group were

of Gleason grade ≥ 7 provided a sobering and thought-provoking counterpoint to the reduction in overall prostate cancer incidence.[4,71]

The SELECT study, designed to test the effect of selenium and vitamin E on prostate cancer incidence, differs from the PCPT primarily in not having an enrollment period in which all participants received a placebo and in not requiring an end-of-study prostate biopsy. In addition, it uses community standards of medical care in diagnosing the endpoint of prostate cancer; that is, within-study biopsies are not mandated and are performed only at the discretion of the treating physician.[7] Moreover, it differentiates by race in its enrollment criteria, reflecting established racial differences in prostate cancer incidence, by permitting African-American men to begin enrolling at ≥ 50 years, whereas others could begin at ≥ 55 years of age. Enrollment in this randomized, placebo-controlled, double-blinded study began in 2001, and its findings are anticipated after the study ends in 2013.[7]

Having reviewed these two major studies, we proceeded then to design a randomized, double-blinded, placebo-controlled chemoprevention trial targeted toward patients with nonmetastatic prostate cancer who had undergone RT.

STUDY DESIGN CONSIDERATIONS

Chemopreventive Agent

There were no special considerations involved in our selection of vitamin D_5; it has been under active and collaborative investigation between investigators at the University of California at Davis and the University of Illinois at Chicago.[16] Vitamin D_3, the parent analogue, has been used in several small clinical trials, although some of them have had to be limited because of hypercalcemia, the major obstacle to the use of vitamin D_3 in pharmacologic doses. These studies on vitamin D_3 or its analogues, which began in 1995,[72] have tried different dosing paradigms or have used vitamin D_3 in combination with other drugs in an effort to reduce its dose[73,74] and minimize hypercalcemia. Our main criteria for selecting the analogue vitamin D_5 were its antiproliferative and differentiation-inducing activities, coupled with its nontoxicity. Because these criteria had been well documented in preclinical cell culture studies and because any toxicity in rats and beagles was not apparent until at ~ 10 times the planned clinical trial dose of 10 μg/day,[16,70] we considered its use in this context to be safe. Moreover, we incorporated dose de-escalation criteria into the trial design to overcome concerns regarding adequate protection of any patient developing symptoms of toxicity.[70]

Target Population

Traditionally, the ideal population in a chemoprevention phase I or II trial for an hitherto untested, but minimally toxic, drug would be those seeking tertiary protection after some type of "curative" therapy. It may also be of benefit if it is given before the curative treatment. However, it would not be used in patients with metastatic cancer because the window of opportunity for chemoprevention is no longer present (unless a curative therapy were available and was planned to be used). Similarly, because of uncertainty in pharmacokinetics, pharmacodynamics, and toxicity in humans, its use may be inappropriate in patients in the primary prevention category (healthy general population) and is perhaps only marginally acceptable for use in those in the secondary prevention category (healthy but at high risk). Because patients in both of these categories have yet to be diagnosed with cancer, administration of drugs known to be toxic, of unknown toxicity, or even of mild toxicity can be open to ethical challenge, given that the potential benefit or benefits to these patients is unclear. In attempting to appropriately address these considerations, we decided to enroll only patients who fell into one specific category: those who had had their prostate cancer treated "curatively" by RT and thereafter needed chemoprevention to prevent or delay the onset of new cancers, recurrences, or biochemical failure.[70]

Patients with prostate cancer have generally been stratified into low-, intermediate-, or high-risk cohorts for biochemical failure or cancer recurrence on the basis of prognostic factors, such as disease stage, PSA level, and Gleason score at initial disease presentation.[75] In one study using these criteria in patients who had undergone RT, the 5-year PSA relapse-free survival was ~ 60% in the intermediate-risk group and ~ 40% in the high-risk group.[65] As the impact of race on prostate cancer incidence became readily apparent, more recent stratifications have included ethnicity in addition to the Gleason score, PSA level, and pathological stage at the time of presentation, to stratify patient risk into very-low-, low-, high-, or very-high-risk categories.[67] In patients who have undergone RP, these authors then calculated 85%, 66%, 51%, and 21%, 7-year disease-free survival in the very-low-, low-, high-, and very-high-risk groups, respectively. Thus, inasmuch as ~ 30%–50% or more of the patients who elect to undergo RT or RP, especially those in the intermediate- and higher-risk groups, may demonstrate either biochemical or clinical failure of their "curative" therapy within 5–7 years, any effective chemopreventive agent that decreases these percentages would be a valued addition to treatment options.

This targeted population of patients with prostate cancer may also obtain an added benefit from their trial

participation with the use of hormonal therapy after RT, that is, at the time of PSA relapse. Such therapy has been shown to improve 5-year disease-specific and biochemical disease-free survival.[76,77] However, the major concern with beginning early hormonal therapy is the increased risk of earlier development of a hormone-refractory state, especially after the development of metastases. Thus, although hormonal therapy may improve metastasis-free survival, patients may actually be hormone refractory when metastases do develop.[78] Moreover, there is often only a short period between PSA recurrence, bone metastases,[79] and detection of occult nodal disease by scintigraphy.[80] Thus, it could be argued that because of the more intensive monitoring of PSA parameters, the fitting of these parameters into the ASTRO[81] and/or Jani et al[82] criteria, and the pre- and poststudy biopsies, we, and the patients in the trial, will be better positioned to

- Detect any occult prostate cancer in the trial participants
- Determine more precisely the optimal time for the initiation of hormonal therapy

Because the ideal PSA thresholds for initiating delayed hormonal therapy have yet to be established,[78] this study may provide exciting new information that will permit the pinpointing of an appropriate time to begin hormone therapy and potentially provide the parameters for the design of clinical trials involving hormone therapy. Moreover, trial participants may benefit by being able to start hormone ablation therapy at a more appropriate point in the disease timeline, possibly contributing to their longer survival and perhaps decreasing the likelihood of developing a hormone-resistant state.

Study Design and Duration

The simplest clinical study design is the randomized one-way layout, in which one study arm is compared individually against another study arm.[8] In our case, having only two randomized arms—vitamin D_5 and placebo—the one-way layout was therefore an appropriate design choice. In addition, because vitamin D_5 has not demonstrated any toxicity except beginning marginally at ~ 10 times the experimental daily dosage used here, any potential toxicity at the experimental dosage could most likely be considered nominal. We elected therefore to combine both phase I and phase II into one randomized phase I/II trial to assess the toxicity, pharmacokinetics, pharmacodynamics, and treatment efficacy of vitamin D_5 in prostate cancer chemoprevention.

A major strength of this study lies in its use of randomization. Randomization eliminates selection biases and allows application of various parametric and nonparametric statistical tests to be applied to the results that will be obtained. In addition, unknown prognostic factors can be better controlled.[8]

To assess and ensure patient compliance, we have incorporated a prerandomization "run-in" period as was also used in the PCPT trial.[3] During this 1-month period, all enrolled patients will take the placebo and keep a pill calendar/diary, which, together with the medication containers, will be carefully monitored during the once-weekly clinic visits. Any degree of compliance totaling < 90% over the month will be grounds for excluding the patient from the study.

After randomization, patients will be monitored medically once a week for 1 month, transitioned to monthly monitoring with weekly telephone calls if this is clinically appropriate and thereafter moved to once every 4 months (Fig. 1).

Biomarkers for Toxicity

The primary toxicity of vitamin D_3 lies in its ability to induce hypercalcemia. This is a major concern in the use of both vitamin D_3 and its analogues.[16] Although vitamin D_5 has not thus far demonstrated hypercalcemia at the doses planned for use in this study, assessing drug toxicity represents a major portion of any phase I study. To that end, serum chemistries, serum albumin, parathyroid hormone (PTH), urine chemistries, and patient questionnaires regarding symptomatology will be closely followed weekly, monthly, and then every 4 months.

Because vitamin D_3 is fat soluble, and the same could be expected of its analogue vitamin D_5, any toxicity may not be apparent until its stores in the body fat have accumulated sufficiently. This formed the basis of our rationale for the 2-year treatment and follow-up phase. This also was a reason for selecting a somewhat healthier cancer patient population for this study because it is

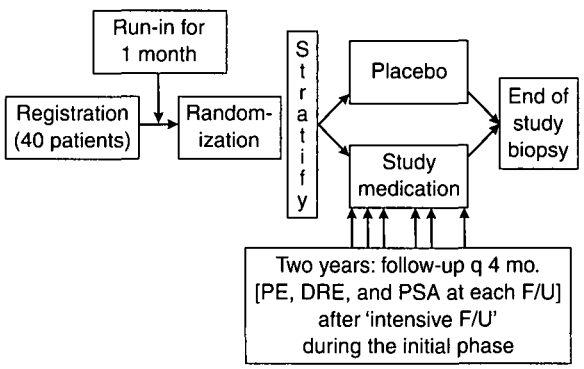

FIGURE 1 Flow sheet depicting timeline, monitoring intervals, and monitored parameters for the vitamin D_5 clinical trial.

highly unlikely that participants with metastatic cancer will survive to provide at least 2 years of data accumulation necessary to demonstrate drug safety for use in primary chemoprevention trials.

Step-wise dose de-escalation protocols for reducing the daily dosage from 10 to 5 to 2.5 μg/day have been incorporated for use in patients demonstrating toxicity via serum chemistries or symptom diaries. Appropriate parameters for withdrawal of a patient from the study because of undue toxicity or other clinically valid reasons have also been included.

Endpoint and Surrogate Endpoint Biomarkers

Several endpoints will be monitored in this study, including vitamin D_5 toxicity, changes in vitamin D receptor number and distribution, biochemical failure indicated by three consecutive increases in PSA,[81,83] biochemical failure as defined by Jani et al,[82] incidence of cancer in the beginning- and end-of-study biopsies, presence of metastatic cancer, QOL assessment, and surrogate biomarker profile.

Potential SEBs that will be assessed in this study include PSA and its associated parameters, such as PSA velocity and doubling time. We also plan to use the prostate biopsy samples as a source of tissue and molecular markers that may potentially function as SEBs. These will include the various grades of PIN; Gleason score; chromosomal markers, especially in the 8q region; molecular markers of apoptosis, such as *Bcl-2, Bax, Bcl-x, PTEN*, and *AKT*; newer molecular markers with prognostic potential, such as *Ki-67*, thymosin-β15, fatty acid synthase, and E-cadherin; and QOL parameters. Potentially, the DNA, RNA, and complementary DNA derived from the tissue samples will be amenable to high-through-put screening techniques using array systems, as has already been demonstrated by other investigators.[84-87] Thus, in correlating the SEBs with the actual endpoint of prostate cancer recurrence or biochemical failure, we anticipate the day when a panel of SEBs or a single SEB may be deemed comparable to an actual endpoint for clinical purposes by the FDA.

Statistical Modeling

The statistical analyses will derive from accepted methodologies under the guidance and expertise of a faculty statistician. Comparisons will be made between each arm using Fisher's exact test for quantitative data with "intent-to-treat" analyses. Nonparametric data will be assessed using Wilcoxon rank sum or log-rank sum, as appropriate. For the QOL assessments, performed via questionnaires every 4 months, we will fit previously described regression models for longitudinal data.[88]

The number of patients we plan to enroll allows sufficient statistical power to detect a decrease in prostate cancer recurrence from ~ 50% in the placebo group to ~ 10% in the vitamin D_5 treatment group. Correlation between longitudinal measures of potential SEBs and prostate cancer recurrence will be assessed in several ways. We will examine differences in time to recurrence using survival models with a time-varying covariate. In addition, we will use repeated measures regression models[88] to determine whether the approach based solely on using change in SEB over time can predict recurrence of disease and can distinguish between the recurrence rates in the two groups. We will test the ability to distinguish recurrence rates by including an indicator term in the model for recurrence; other, more complex, statistical models will examine time to recurrence or whether different recurrence rates in the two groups could be detected. As we have noted previously, the statistical modeling and validation of SEBs are a mathematically complex and demanding endeavors. Readers are referred to the references cited previously[58,59] for more details regarding these statistical analyses.

Summary

To summarize, in our randomized, placebo-controlled phase I/II chemoprevention clinical trial design, we anticipate recruiting 40 patients (20 for each arm) who are all within ~ 12–60 months of completion of RT for prostate cancer. They will be randomly assigned to either the D_5 or the placebo arm after 1 month of placebo administration (the run-in period) to assess the quality of patient compliance. All patients will undergo a pretreatment biopsy, receive baseline clinical staging, and undergo serum PSA level measurement. Serum chemistries, serum albumin, serum PTH, and urine electrolytes will also be measured. For the first month at least, all subjects will be monitored weekly via serum chemistries and albumin levels. Thereafter, individuals who continue to demonstrate stable and nonhypercalcemic serum calcium levels will be monitored with weekly phone calls and continue with monthly clinical and laboratory assessments of serum chemistries, albumin levels, PTH levels, and urine electrolyte levels. Individuals who continue to demonstrate stable serum calcium levels at 4 months will then transition to a 4-month monitoring cycle with biannual measurements of serum PTH level (see flow sheet in Fig. 1).

We anticipate being able to monitor all the study participants for a minimum of 2 years for the trial except in the event of patient death or medically justifiable inability to continue in the protocol or patient's voluntary withdrawal from the trial. However, routine and extended follow-up care will continue as long as the enrollees remain patients of the University of California at Davis Cancer Center.

The strengths of this trial lie in its randomization and

placebo control, the optimization of target population selection, the assessment of SEBs, and its use of end-of-study biopsies to confirm and provide correlative evidence of outcomes. We hope that this combined phase I/II trial will serve as an useful model for small, efficient clinical trials that assess chemopreventive potential as well as accumulate valuable data on the use of SEBs.

CONCLUSION

In this paper, we have briefly considered some of the core concepts underlying chemoprevention, clinical trials, endpoints, and SEBs. Of the myriad forms and types of cancer facing our patients, we elected to direct our attention primarily to the goal of prostate cancer chemoprevention. To that end, we have discussed herein our experience in designing an institutional review board–approved, small, selective, combined phase I/II randomized, placebo-controlled, double-blinded clinical trial, that uses vitamin D_5 and may serve as a model for data accumulation about selected SEBs and cancer recurrence. Importantly, the study includes end-of-study biopsies that all participants undergo to ensure that tissue samples are available for correlation with the SEBs used in the study, as well as for the analysis of newer genetic/molecular markers that could potentially serve as SEBs. To accomplish these aims, we elected to obtain study participants from a high-risk population of patients with prostate cancer who, because of poorer prognostic factors on initial presentation, may face a higher incidence of biochemical failure or cancer recurrence after RT. We anticipate that the toxicity and clinical data gathered herein, as well as in the other studies using vitamin D and its analogues, will accelerate the day when vitamin D_5 will become available as an effective and safe chemopreventive agent for all men.

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Identification of novel RARβ2 transcript variants with short 5′-UTRs in normal and cancerous breast epithelial cells

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Functional significance of RARβ2 as a putative tumor suppressor gene has been studied in breast cancer and other tumors. The long 5′-untranslated region (5′-UTR) of its transcript with multiple open-reading frames (uORFs) is considered as a regulatory unit for translation. Here, for the first time we identified RARβ2 transcript variants with short 5′-UTRs in both normal and malignant breast epithelial cells. The 5′-RACE analysis of RARβ2 mRNA in these cells demonstrated the existence of short RARβ2 transcript variants that are identical to the sequence of known RARβ2, but lack all the uORFs present in the full-length 5′-UTR. By RT–PCR analysis, we found that the expression of both transcripts with short and full-length 5′-UTR is mediated by retinoic acid, while cellular sensitivity is preferentially correlated to upregulation of short RARβ2 transcript variants in response to retinoic acid. The transfection and *in vitro* translation assay indicated that the short 5′-UTR has no inhibitory effects on translation, while the presence of full-length 5′-UTR inhibited translation by 60%. In addition, no promoter activity was detectable in RARβ2 full-length 5′-UTR region. Our data suggest that the RARβ2 transcript variants with short 5′-UTR may serve as major transcripts for RARβ2 protein translation as well as potential targets for retinoids in breast cancer prevention and therapy studies.

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The clinical significance of retinoids in prevention and treatment has been well established for the cancers of many target organs including breast (Tallman and Wiernik, 1992; Lippman *et al*., 1995; Lotan, 1996). The biological effects of retinoids are mainly mediated by retinoic acid receptors (RARs and RXRs), each consisting of three receptor subtypes (α, β, γ) (Giguere, 1994; Chambon, 1996). As the primary effectors of retinoid signaling, the RARs and RXRs themselves appear to be targets for disruption in tumorigenesis (Chen *et al*., 2002). For example, the RARβ expression is lost during carcinogenesis, suggesting that it may play a role in tumor suppression (Deng *et al*., 1996; Widschwendter *et al*., 1997; Xu *et al*., 1997). So far the identified human RARβ2 transcript has a long 5′-untranslated region (5′-UTR, 468 nucleotides) that can form stable secondary structures and contains five small open-reading frames (uORFs) preceding the major ORF (de The *et al*., 1987; Zelent *et al*., 1991). The uORFs present in the 5′-UTR usually inhibit translation from downstream (Gray and Wickens, 1998) and therefore they may greatly decrease the translation efficiency of RARβ2. However, it has also been reported that the uORFs of RARβ2 mRNA plays a role in the control of tissue-specific and developmentally regulated gene expression (Zimmer *et al*., 1994).

We have been interested in RAR/RXR alterations in the process of breast tumor progression using MCF10 series of breast epithelial cell lines as a model system (Santner *et al*., 2001; Peng *et al*., 2004b). During the characterization of RARβ expression in these cell lines, we found that RARβ2 transcript was not detectable by PCR with primers designed based on its 5′-UTR sequence; further analysis has led to the identification of novel RARβ2 transcripts containing short 5′-UTRs that exhibited higher translation efficiency in breast cancer cells. These results indicate that the previously identified RARβ2 transcript with long 5′-UTR could represent less functional form of RARβ2.

In order to characterize the 5′ region of RARβ2 in MCF10 series of cell lines, we performed RT–PCR analysis with total mRNA isolated from these cell lines using two primer pairs specifically designed for different region of RARβ2. As shown in Figure 1a, we detected the expression of RARβ2 mRNA with primers RARβ2-475F (number identifies the primer location relative to transcription start site (TSS) based on GeneBank RARβ sequence NM_000965) and RARβ2-730R. However, we did not detect RARβ2 when the forward primer was replaced with RARβ2-110F (this primer pair is supposed to amplify both RARβ2 and RARβ4). RT–PCR analysis with another primer pair (RARβ2-79F and RARβ2-401R) confirmed the results (data not shown), suggesting that MCF10 series of cell lines expressed RARβ variants with different 5′-end. In order to

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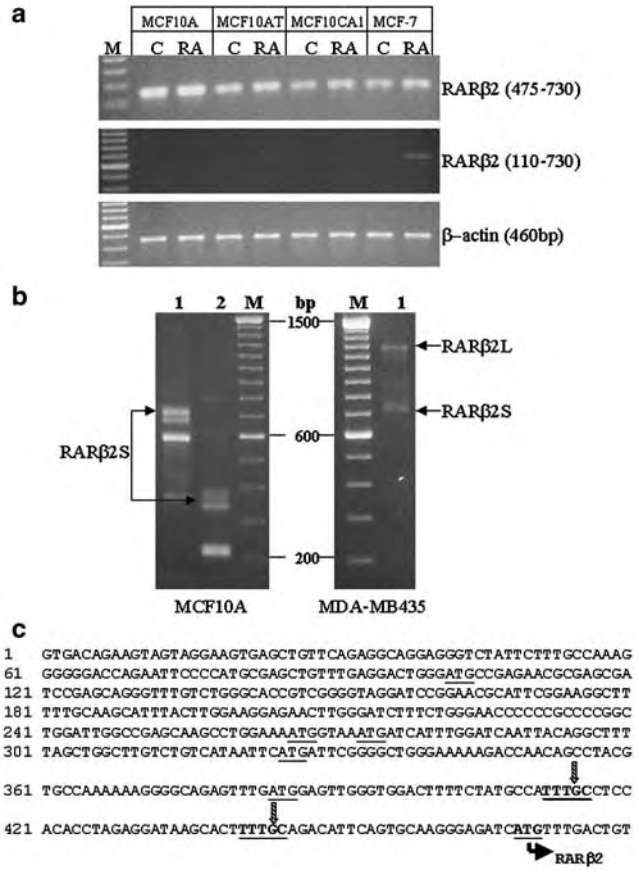

Figure 1 Identification of RARβ2 transcript variants. (**a**) MCF10A series of cell lines and MCF-7 cells express RARβ2 transcript variants. Cells were treated with DMSO (C) or 1 μM atRA (RA) for 48 h, and then total RNA was subjected to RT–PCR analysis with different sets of RARβ primers (475FP, GACTGTATGGATG-TTCTGTCAG; 730RP, ATTTGTCCTGG CAGACGAAGCA; 110FP, AACGCGAGCGATCCGAGCAG). Note there was no detectable product in MCF10A series of cell lines for primers RARβ2-110F and RAR β2-730R. M, Maker. Actin primers: forward, GTCACCAACTGGGACGACA; reverse, TGGCCATCTCTTGCTCGAA. (**b**) 5′-RACE analysis of MCF10A and MDA-MB435 mRNA with SMART RACR cDNA amplification Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) according to the User Manual. For MCF10A cells, two primer pairs, UPM (universal primer mix, provided with kit)-RARβ2-1104R (TCGGACTCGATGGTCAGCACTGGAA) (lane 1) and UPM-RARβ2-727R (TGTCCTGGCAGACGAAGCAGG GTTT) (lane 2) were used for analysis, while for MDA-MB435, one primer pair UPM-RARβ2-1104R (lane 1) was used for analysis. In MCF10A cells, the full-length RARβ2 (expected size: 1156 bp for primers UPM-RARβ2-1104R (lane 1); 779 bp for primers UPM-RARβ2-727R (lane 2)) was not detectable, but two bands at ~720 bp (for primer pair UPM-1104R, arrow) was consistently detectable for both primers pairs (lanes 1 and 2). In MDA-MB435 cells, both full-length RARβ2 (size ~1.15 kb) and a band at ~720 bp were detectable. M, DNA marker. (**c**) 5′-UTR cDNA sequence of full-length RARβ2 transcript. Nucleotides are numbered relative to the TSS of full-length RARβ2 mRNA. The 5′-uORFs' start codons (uATG) were underlined. The sequences surrounding the alternative TSS for the transcript variants in MCF10A cells (see Table 1) were underlined and the TSS were indicated above the arrow. Translation of RARβ2 begins at +469 of full-length RARβ2 transcript, indicated above the right-angled arrow

distinguish between multiple 5′-ends, we performed 5′-RACE analysis of the MCF10A total RNA with two RARβ2-specific primers, RARβ2-1104RP and RARβ2-727RP, respectively (Figure 1b). Using these two primers, we failed to detect the expected RARβ2 fragments with size ~1.15 kb (Figure 1b, lane 1) and ~0.78 kb (Figure 1b, lane 2), respectively, but consistently detected two bands with smaller sizes (~0.45 kb shorter, Figure 1b, lanes 1 and 2). We further 5′-RACE-analysed MDA-MB435 cells that expressed high level of RARβ2 (Sommer *et al*., 1999) with primer RARβ2-1104RP. We detected both expected RARβ2 fragment with size of ~1.15 kb (labeled as RARβ2L in Figure 1b) and a smaller fragment with size of ~720 bp (labeled as RARβ2S, ~0.45 kb shorter).

The 5′-RACE product was cloned and sequenced. Novel RARβ2 transcript variants with short 5′-UTRs were identified. Table 1 lists the 5′-RACE mapping of the transcriptional start sites (TSS) of these transcript variants. In order to confirm the accuracy and effectiveness of 5′-RACE mapping of TSS, we also analysed the RARβ2 fragment of ~1.15 kb (RARβ2L from Figure 1b) with the same method. Two TSS (+1 and −11; +1 identifies the first nucleotide of the putative TSS based on GeneBank sequence data (NM_000965)) of the full-length RARβ2 were identified in MDA-MB435 cells. At the present time, we have analysed two clones. It is possible that multiple TSS of RARβ2 may be present and remains unidentified in this breast cancer cell line. Correspondingly, multiple TSS were identified for RARβ2 transcript variants with short 5′-UTR from MDA-MB435 cells, whereas only two TSS (+444 and +415) were identified for RARβ2 variants from MCF10A cells, corresponding to the two bands identified by 5′-RACE analysis in this cell line (Figure 1b, lanes 1 and 2). Interestingly, the DNA sequences flanking the two TSS of RARβ2 variants from MCF10A cells are the same; both are TTTGC (Figure 1c). Same TSS for RARβ2 variant was also identified from normal mammary epithelial cell (HMEC) (Table 1). As shown in Figure 1b, the lower

Table 1 Transcriptional start site mapping (5′-RACE) of hRARβ2 mRNA in MCF10A mammary epithelial cells, MDA-MB435 breast cancer cells and normal HMEC

	Transcriptional start sites of RARβ2 variants		
Clone	*MCF10A*	*MDA-MB435*	*HMEC*
1	415	441	444
2	444	444	
3	415	444	
4	444	438	
5	444	415	
6	415	444	
7	444	421	
8		444	

Note: The nucleotide position of hRARβ2 mRNA is designated with +1 identifying the first nucleotide of the putative transcriptional start site based on GeneBank sequence data (X51650, NM_000965, M96016)

brighter band below the RARβ2S bands was recently identified by our group as a novel RARβ isoform, RARβ5 (Peng *et al*., 2004a).

To detect quantitatively the expression of full-length RARβ2 transcripts and total RARβ2 transcripts, real-time RT–PCR was performed with RARβ2-specific primers. Although MCF10A series of cell lines express short RARβ2 transcripts only and are good model for the identification of these short transcripts, it appears that RARβ2 transcripts were not significantly mediated by atRA (Figure 1a). Therefore, we selected ER-positive T47D and ER-negative MDA-MB435 breast cancer cell lines to further address the regulation of both full-length and short transcripts. As shown in Figure 2, both full-length RARβ2 transcript and total RARβ2 transcripts were significantly upregulated by atRA in both ER-positive T47D and ER-negative MDA-MB435 cells. In T47D cells, atRA treatment increased the expression of full-length RARβ2 transcript by 17-fold, whereas increased total (full-length plus short) RARβ2 transcripts by 41-fold; on the other hand, in MDA-MB435 cells, atRA increased full-length RARβ2 transcript by 14-fold and increased total RARβ2 transcripts only by 8-fold (Figure 2a). These results suggest that both full-length and short RARβ2 transcripts were upregulated by atRA in these cell lines; in T47D cells, short RARβ2 transcripts were preferentially upregulated, whereas in MDA-MB435 cells, full-length RARβ2 transcripts were preferentially upregulated. The ratio analysis of total RARβ2 transcripts to full-length RARβ2 transcripts (total/full length) confirmed this result (Figure 2b, the change of ratio roughly reflects the change of short transcripts), since the ratio was greater than control in T47D cells and lower than control in MDA-MB435 cells after atRA treatment. The effect of atRA on cell proliferation was evaluated by MTT assay (Carmichael *et al*., 1987) in these two cell lines. As shown in Figure 2c, 7-day treatment with 1 μM atRA significantly inhibited cell proliferation by 40% in T47D cells, but failed to inhibit cell proliferation in MDA-MB435 cells. This cellular sensitivity to atRA is correlated to the upregulation of short RARβ2 transcripts, suggesting that the expression of short RARβ2 transcripts could contribute to cellular sensitivity.

To test whether short RARβ2 transcripts are more functional, we further accessed the effects of long and short 5′-UTR on translation efficiency. Full-length (−3/+468) and short (+415/+468) 5′-UTRs of RARβ2 transcripts were cloned into the upstream of luciferase ORF of PGL3C vector (Figure 3a). Transient transfection assay demonstrated that the presence of full-length 5′-UTR inhibited luciferase translation by 60% in both MCF-7 and T47D breast cancer cell lines (Figure 3b), whereas the short 5′-UTR had no inhibitory effects in both cell lines as compared to the translation efficiency of the control vector PGL3C. To confirm these results, we also cloned the two 5′-UTRs to the upstream of RARβ2 ORF in the pcDNA3.1(RARβ2) expression vector and performed *in vitro* translation with these expression vectors containing different 5′-UTRs. Similar results were obtained (Figure 3c). These results suggest

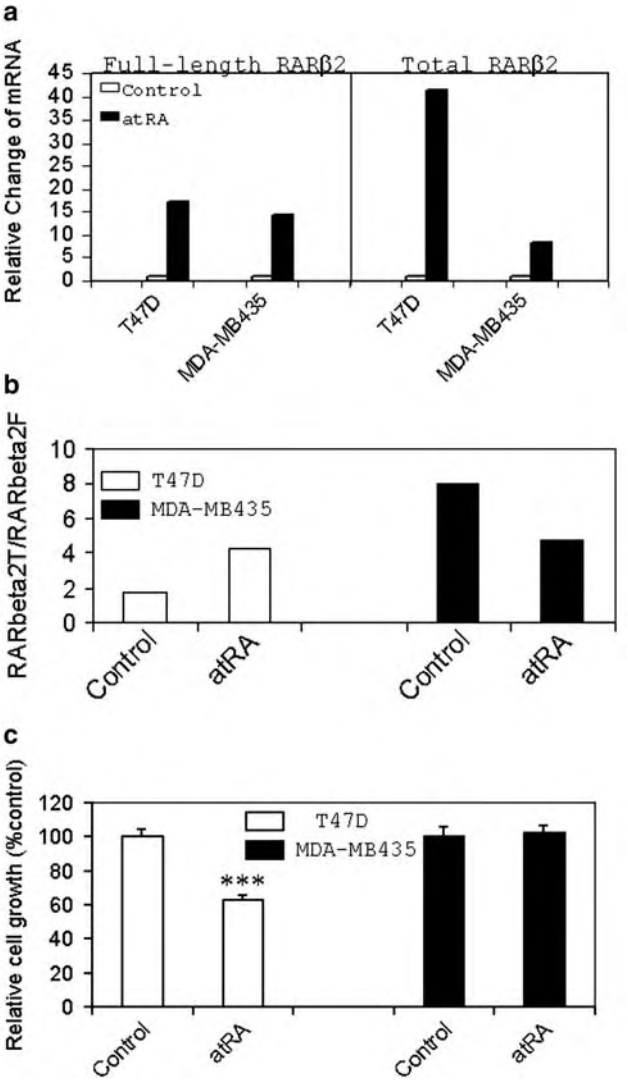

Figure 2 RT–PCR analysis of full-length RARβ2 transcript and total RARβ2 transcripts in breast cancer cells in correlation to cellular response to atRA. T47D and MDA-MB435 cells were treated with atRA (1 μM) for 24 h, total RNA was then analysed by quantitative RT–PCR with 1 μl RT product using 7900HT Sequence Detection System (ABI, Applied Biosystem) and ABI 2 × SYBR Green PCR Master Mix (ABI #4309155) according to ABI's recommended guidelines. (**a**) Real-time PCR analysis of the cDNA samples from the two cell lines showing relative changes of full-length RARβ2 transcript and total RARβ2 transcripts normalized to β-actin (their controls are set as 1) after atRA treatment. Results are expressed as the mean value of duplicate experiments. Primers for real-time PCR for total RARβ2 transcripts were 584FP (GATTGACCCAAACCGAATGGCAGCA, Tm = 66C) and 730RP; primers for full-length RARβ2 were 295FP (GGCTTTTA GCTGGCTTGTCTG) and 420RP (GGAGGCAAATGGCATA GAAA). (**b**) Real-time PCR analysis showing the change of ratio of total RARβ2 transcript (RARβ2T) to full-length RARβ2 (RARβ2F), which roughly represents the change of short 5′-UTR RARβ2 transcripts. (**c**) MTT assay of cell proliferation in response to atRA. Cells were treated for 7 days with 1 μM atRA. Data are expressed as the percentage of DMSO control ± s.d. of eight wells. All data shown are representative of three independent experiments. ***$P<0.001$ compared to control. Note the correlation between cellular sensitivity and the ratio of total RARβ2 transcript to full-length RARβ2 in response to atRA

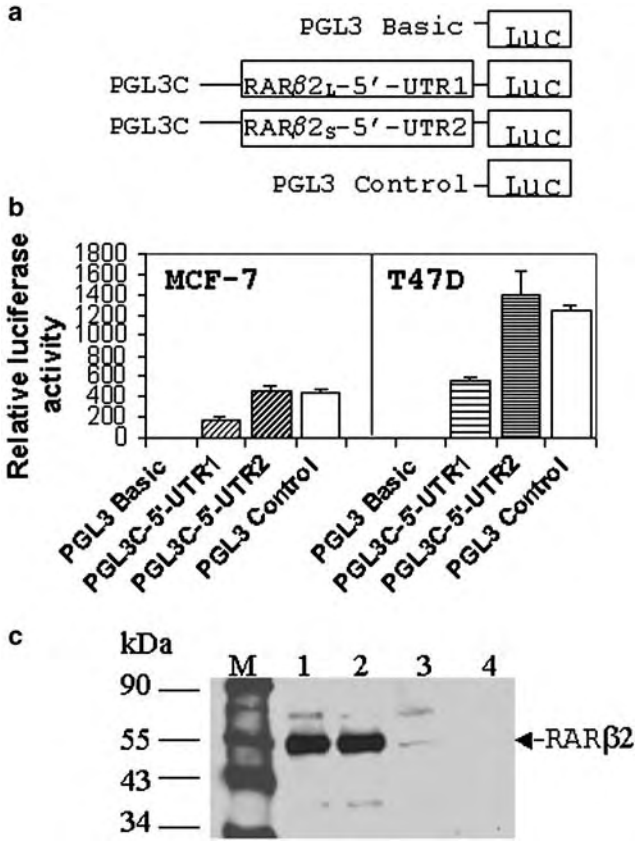

Figure 3 Effect of different 5′-UTRs of RARβ2 on translation efficiency. (**a**) Schematic representations of the 5′-UTR-luciferase constructs. (**b**) Luciferase assay of the effect of 5′-UTRs of RARβ2 on translation efficiency in MCF-7 and T47D cells. Luciferase reporter constructs with full-length (PGL3C-5′-UTR1) or short (PGL3C-5′-UTR2) 5′-UTR of RARβ2 were cotransfected with β-gal vector into MCF-7 and T47D cells. After 24 h incubation, cells were lysed and luciferase activity was assayed. Relative luciferase activity, luciferase activity after normalized to β-gal (luciferase activity of PGL3 basic vector-transfected cells was set as 1). Results are expressed as mean ± s.e.m. of three independent experiments performed in triplicate. PGL3 control was also included for positive control assay. (**c**) Effect of different 5′-UTRs of RARβ2 on RARβ2 *in vitro* translation efficiency. 5′-UTRs of RARβ2 were cloned to the upstream of RARβ2 ORF of pcDNA3.1(RARβ2) expression vector. Then, *in vitro* translation was performed with different pcDNA3.1(RARβ2) constructs containing full-length or short 5′-UTR of RARβ2. *In vitro* translation products were subjected to Western blot analysis with RARβ specific antibody (sc-552, Santa Cruz). M, protein marker; 1–4, *in vitro* translation product with 1. pcDNA3.1(RARβ2) expression vector; 2. pcDNA3.1(RARβ2) containing short 5′-UTR; 3. pcDNA3.1(RARβ2) containing long 5′-UTR; 4. pcDNA3.1 empty vector

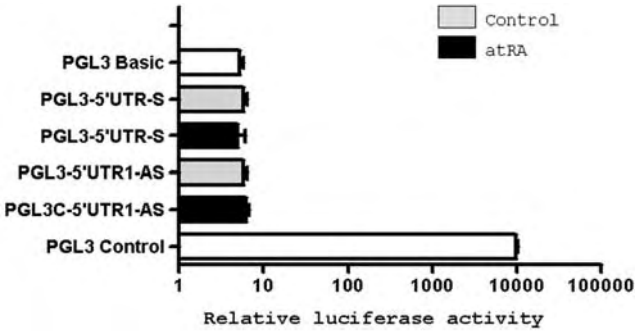

Figure 4 Full-length 5′-UTR of RARβ2 has no promoter activity in MCF-7 cells. PGL3 vectors containing full-length of RARβ2 5′-UTR (−3/+468) in sense (−S) and antisense (−AS) orientation as promoter was cotransfected with β-galactosidase construct into MCF-7 cells. After 5-h incubation with different constructs, cells were treated with atRA (1 μM) for 24 h, and then luciferase activity was assayed. Luciferase activity was normalized to β-gal. PGL3 control vector was used as positive control vector. There were no significant difference in luciferase activity among cells transfected with PGL3 basic vector and other PGL3-5′-UTR constructs. Results are expressed as the mean ± s.e.m. of three independent experiments performed in triplicate

that full-length 5′-UTR greatly inhibited RARβ2 translation, whereas short 5′-UTR had no significant effect on translation efficiency for *in vitro* translation.

Owing to the identification of these RARβ2 transcript variants with short 5′-UTRs and RT–PCR analysis of the regulation of full-length and total RARβ2 transcripts by atRA, we suspected that the 5′ region flanking the short 5′-UTRs of RARβ2 variants could play a role of promoter for the transcription of these variants with short 5′-UTRs. We therefore cloned this region (−3/+468) to the upstream of luciferase ORF of promoterless PGL3 basic vector in both sense and antisense orientation. Transient transfection assay demonstrated that this region has no significant promoter activity in MCF-7 cells (Figure 4), as the luciferase activity did not show any difference from that of empty vector PGL3B-transfected cells in either the presence or absence of atRA stimulation. Similar results were also obtained from T47D cells (data not shown). These results suggest that this region does not play a role as an independent promoter.

RARβ gene was first identified in 1987 (de The *et al*., 1987); in this report, Northern blot analysis of RARβ from different tissues and cells had revealed two RARβ bands (~3 and 2.5 kb), the lower band was not detectable using probe specific to the 5′-UTR region of RARβ, suggesting that the two RARβ transcripts differ at least in their 5′-untranslated leader sequence. These results are consistent with our observations. Similar results have also been reported by others (Swisshelm *et al*., 1994) and demonstrate the presence of two RARβ transcripts. However, the difference of the two transcripts has never been precisely characterized. Here, we provide the first evidence for the existence of multiple RARβ2 transcript variants that only differ in their 5′-UTR and these variants are expressed in both normal and neoplastic breast epithelial cells. It should be indicated that the RARβ2 transcript variants with short 5′-UTR are different from RARβ4, which is a splice variant of full-length RARβ2 and uses CUG as its translation start codon. Therefore, cell lines not expressing full-length RARβ2 such as MCF10A series of cell lines do not express RARβ4 (Figure 1a), while these cell lines still express short RARβ2 transcripts. The identification of these variants raises new questions: Does the 5′-UTR of different transcripts influence the translation efficiency? Whether the short transcripts are generated by alternative splicing or from different TSS.

The first question was addressed through transfection and *in vitro* translation assay. As expected, the short transcripts exhibited highest translation efficiency, whereas the translation efficiency of the full-length transcripts greatly decreased in both cells and *in vitro* translation assay. Upregulation of short transcripts are further correlated with cellular sensitivity, suggesting that the short transcripts could have greater functional significance.

To address the second question, we cloned the 5′-UTR region to promoterless PGL3 basic vector to test its promoter activity. We were unable to detect any promoter activity of this 5′-UTR region. We therefore thought the short transcripts could be formed by post-transcriptional modification (splicing or truncation), since the flanking sequence of the start sites of the two short transcripts from MCF10A cells are the same (TTTGC). However, the analysis of RARβ2 transcripts from MDA-MB435 cells excluded this possibility. The MDA-MB435 cells have multiple start sites for both the short and long transcripts, suggesting that these short transcripts are possibly from different TSS rather than an enzyme modification, because enzyme generally cuts precisely at specific sequence site. Real-time PCR analysis apparently also supports this hypothesis, since the expression of both long and short RARβ2 transcript appeared to be stimulated by atRA in a similar way. Although the 5′-UTR of full-length RARβ2 has no promoter activity, it is still possible that this region together with P2 promoter that has been known to control the transcription of full-length RARβ2 directs the transcription of short RARβ2 transcripts. Our data on MCF10A cells support this hypothesis. In this cell line, P2 promoter controlling the full-length RARβ2 transcripts are silenced, whereas the short transcripts are still expressed, revealing different regulation mechanisms of different transcripts in these cells. We previously detected low level of RARβ2 protein in these cell lines and that the protein expression was also induced by retinoids (Peng *et al*., 2004b); therefore, it is possible that RARβ2 transcript variants with short 5′-UTRs provide basal level of transcripts for RARβ2 protein translation. In agreement with this hypothesis, Sommer *et al*. (1999) reported that MCF-7 and MDA-MB231 breast cancer cells did not express detectable (by Northern blot) RARβ2 mRNA, but expressed detectable RARβ2 protein, while MDA-MB435 cells expressed high level of full-length RARβ2, but did not express high level of RARβ2 protein. Further studies are needed to evaluate the function of the flanking sequence (TTTGC) of the TSS of the short RARβ2 transcripts, which is also conserved in the 5′-UTR close to TSS of mouse RARα2 (Leroy *et al*., 1991) and mouse RARβ2 (Zimmer *et al*., 1994), as well as how the promoter regulate the expression of these RARβ2 transcript variants. It is known that RARβ2 promoter is silenced in some breast cancer cells through methylation (Sirchia *et al*., 2000), but it is still unknown how the promoter methylation influence the expression of different transcripts of RARβ2. We also conducted studies to assess whether deacetylase inhibitor such as trichostatin A (TSA) affects the RA-induced RARβ2 expression in MCF10AT cells. Our data, however, did not show significant alterations of RARβ2 expression in this cell line after TSA treatment, although TSA greatly inhibited cell proliferation (data not shown).

It should be noted that RARβ2 transcripts with short 5′-UTRs could be expressed at low level and might not always be detectable by Northern blot in some cells such as MCF10A (Li *et al*., 1995) and MCF-7 cells (Sommer *et al*., 1999), but detectable by RT–PCR. This may partially explain the mismatch between protein level and RNA level in some cells.

In summary, we have identified novel RARβ2 transcripts with short 5′-UTR that are more functional for protein translation. It appears that their expression is also controlled by P2 promoter and stimulated by atRA. Their upregulation is correlated to cellular sensitivity to atRA. These results provide the basis for further studies on the regulation of these more functional RARβ2 transcripts as a target for breast cancer chemoprevention and treatment with retinoids.

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Chemoprevention of chemically-induced mammary and colon carcinogenesis by 1α-hydroxyvitamin D_5

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Abstract

Epidemiological data as well as experimental models yield evidence for a protective effect of vitamin D against the genesis of several types of cancers. Given its toxic properties at effective concentrations, numerous analogs of vitamin D have been developed. We synthesized an analog of vitamin D_5, 1α-hydroxy-24-ethylcholechalciferol (1α(OH)D_5) and previously reported on its anti-proliferative activities against several cancer cell lines. To further examine its chemopreventive potential, experiments were conducted to investigate the in vivo effects of 1α(OH)D_5 using the *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis model. Results showed that 1α(OH)D_5 (25 and 50 μg/kg diet) decreased the incidence and multiplicity of mammary tumors in female Sprague–Dawley rats. In a subsequent study, the stage specific inhibition was investigated using the 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis model. While supplementation with of 1α(OH)D_5 (40 μg/kg diet) showed no significant effects during the initiation phase, tumor incidence during the promotional stage was significantly ($p < 0.05$) decreased by 37.5%.

In the colon, 1α(OH)D_5 (25 μg/kg diet) was highly effective ($p < 0.001$) in inhibiting the development of azoxymethane (AOM)-induced Aberrant crypt foci (ACF) in CF-1 mice. Studies on the stage specific inhibitory effects of 1α(OH)D_5 in the colon demonstrated that animals receiving 1α(OH)D_5 (25 μg/kg diet) during the initiation, promotion, and entire period had a reduction in ACF number of 71, 80 and 82%, respectively. Immunohistochemistry studies comparing the colons of animals receiving control versus 1α(OH)D_5 supplemented diets showed that 1α(OH)D_5 partly mediates its effects by regulating members of the oncogenic β-catenin pathway. 1α(OH)D_5 inhibited expression of β-catenin and peroxisome proliferator-activated receptor β, a β-catenin-TCF-4 responsive gene, whereas it induced expression of VDR. Cumulatively, these studies support the chemopreventive properties of 1α(OH)D_5 against the development of breast and colon cancers.

1. Introduction

Historical documents dating back to many centuries ago describe vitamin D related diseases such as osteoporosis and rickets [1,2]. However, it was not until the 20th century that science began to unravel the underlying source for these conditions. Vitamin D_3 was identified in the early 1920s as substance with anti-rachitic properties [1,2]. Numerous studies have been undertaken to better understand the cancer chemopreventive actions of vitamin D and its active metabolite, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). To date, vitamin D has been reported to be protective against several types of cancers, including carcinoma of the colon, breast and prostate [3].

Epidemiological data have suggested possible protective effects of vitamin D and its analogs on breast cancer risk. Garland et al. [4] reported that the mortality rates for breast cancer follow a geographical gradient in the United States with the highest rates reported in the northeast part of the country. Moreover, several population studies have shown an inverse association between vitamin D intake and breast cancer risk. In the Nurse's Health Study, total vitamin D intake was inversely associated with pre-menopausal breast cancer risk [5]. Likewise, several variables of sunlight exposure and dietary vitamin D intake have each been shown to be correlated with reduced breast cancer risk [6]. Recent advances in molecular biology have provided further evidence for the

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connection between vitamin D and breast cancer. For example, 25-(OH)D_3-1α-hydroxylase, responsible for 25-(OH)D_3 activation has be detected in both normal breast tissue and in breast carcinoma samples, indicating that both could be capable of 25-(OH)D_3-1α-hydroxylation and local synthesis of 1,25-$(OH)_2D_3$ [7].

In colon cancer, Garland and Garland [8] first reported that in the United States white men exposed to the highest mean solar radiation had the lowest age-adjusted rates of death due to this disease, whereas states with the lowest mean solar rates had the highest number of colon cancer related deaths. Since then, a number of cohort and case-control studies have attempted to elucidate the relationship between vitamin D and colon cancer [9–15]. In a recent review, the strongest support for a protective effect of vitamin D was documented for studies in which total ingested vitamin D came from diet sources as well as from supplements [10]. Seven out of eight studies which used colon or colorectal cancer as the outcome variable reported an odds ratio (OR) or relative risk (RR) of <1.0. It should be noted that when dietary vitamin D was used as the sole variable of interest, the association between vitamin D and colon cancer was found to be less significant. In this review, four of the five case-control studies were found to have an odds ratio of <1.0, but only two studies had a 95% confidence interval of <1. Similar results were reported for cohort studies of dietary vitamin D and colon cancer. One explanation for the inconsistencies observed within population studies may be the inability to accurately measure vitamin D intake [10]. Given that, recent studies have used serum 25-(OH)D_3 as a biomarker for vitamin D status. The use of serum 25-(OH)D_3 represents the sum of vitamin D from diet, supplements, and exposure to solar and artificial radiation. Using this biomarker, Platz et al. [16] found an association between low plasma levels of 1,25-$(OH)_2D_3$ and 25-(OH)D_3 with an increased incidence of polyp formation in the distal colon of women. Similarly, in a case-control study adenoma risk decreased by 26% with each 10 ng/mL increase in serum 25-(OH)D_3 [17].

2. Vitamin D and cancer chemoprevention

In 1976, Sporn coined the term “chemoprevention” and since then it has been an active area of research [18]. Conceptually, chemoprevention of cancer has been defined as an intervention in the carcinogenic process by an agent which is derived from natural products [19] or synthetic in nature. In a broad sense, chemoprevention of cancer is a mode of cancer control in which formation of the disease can be prevented or disease latency can be significantly delayed. In practice, this can best be achieved by the dietary administration of chemical agents that can enhance the physiological processes that protect the organism against the growth of preneoplastic or cancer cell growth. Chemoprevention of cancer focuses on two categories of a population. One of these is the healthy population, and the other is a group of people who are at a higher risk of developing cancer (Fig. 1). Individuals in the general population certainly are exposed to environmental carcinogens and are at risk of developing cancer; however, they are currently disease-free. The most prudent way of chemoprevention in healthy people is by intelligent selection of foods or supplements [19]. On the other hand, people at high risk of developing cancer may reduce the risk by selection of chemopreventive foods as well as by chemopreventive agents that may be purely synthetic. Many compounds belonging to diverse structural and functional chemical classes have been identified as potential chemopreventive agents [20]. For example, agents that induce differentiation such as vitamin D have shown promise as chemopreventive agents [21]. In light of this, attempts have been made to synthesize less toxic vitamin D analogs that would exhibit a reduced hypercalcemic response while retaining its chemopreventive and chemother-

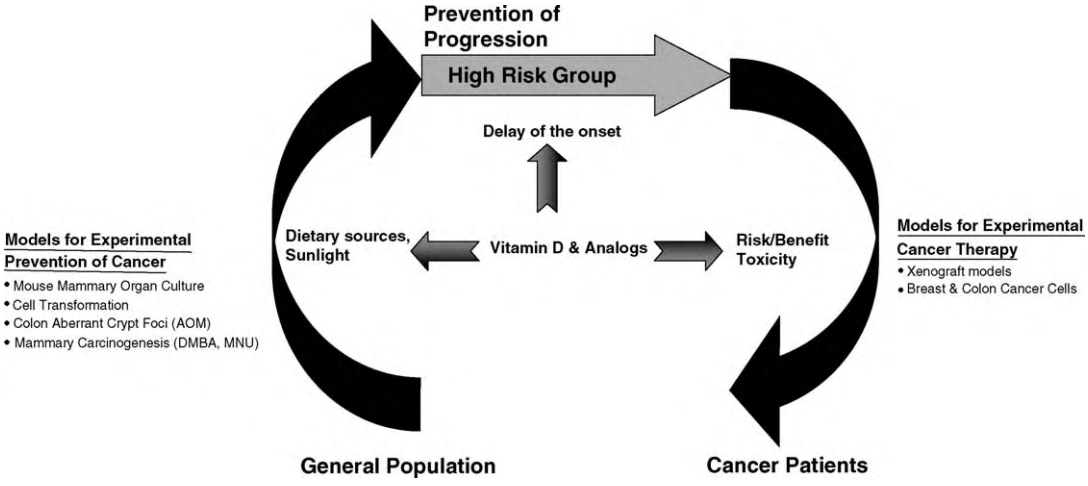


Fig. 1. Schematic diagram illustrating the potential applications for vitamin D and its analogs among different population groups.

apeutic properties. Over 1000 analogs of vitamin D have been synthesized.

3. Synthesis and organ specificity of 1α(OH)D_5 in cancer

We synthesized an analog of 24-ethylvitamin D_3 (vitamin D_5 series), i.e., 1α-hydroxyvitamin D_5 (1α(OH)D_5) (Fig. 2). The synthesis of this compound has been previously described [22]. Since then, 1α(OH)D_5 has been evaluated using several target organs, including cancer models for breast, prostate, melanoma and colon (Fig. 3). The results have been promising with the exception of melanoma, where 1α(OH)D_5 was found to be inactive. Previously, we reported that 1α(OH)D_5 inhibited development of estrogen- and progesterone-dependent ductal lesions as well as steroid hormone-independent alveolar lesions in a mammary gland organ culture (MMOC) model. Moreover, the inhibitory effect of 1α(OH)D_5 was more significant during the promotional phase of the lesion development. The growth inhibitory effect of 1α(OH)D_5 has also been manifested in several breast cancer cell lines, including BT-474 and MCF-7. Breast cancer cell lines that responded to 1α(OH)D_5 treatment were vitamin D receptor positive (VDR+). Vitamin D receptor-negative (VDR−) cell lines, such as MDA-MB-231 and MDA-MB-435, did not show growth inhibition upon incubation with 1α(OH)D_5 suggesting the requirement of VDR in 1α(OH)D_5-mediated growth effects [23,24].

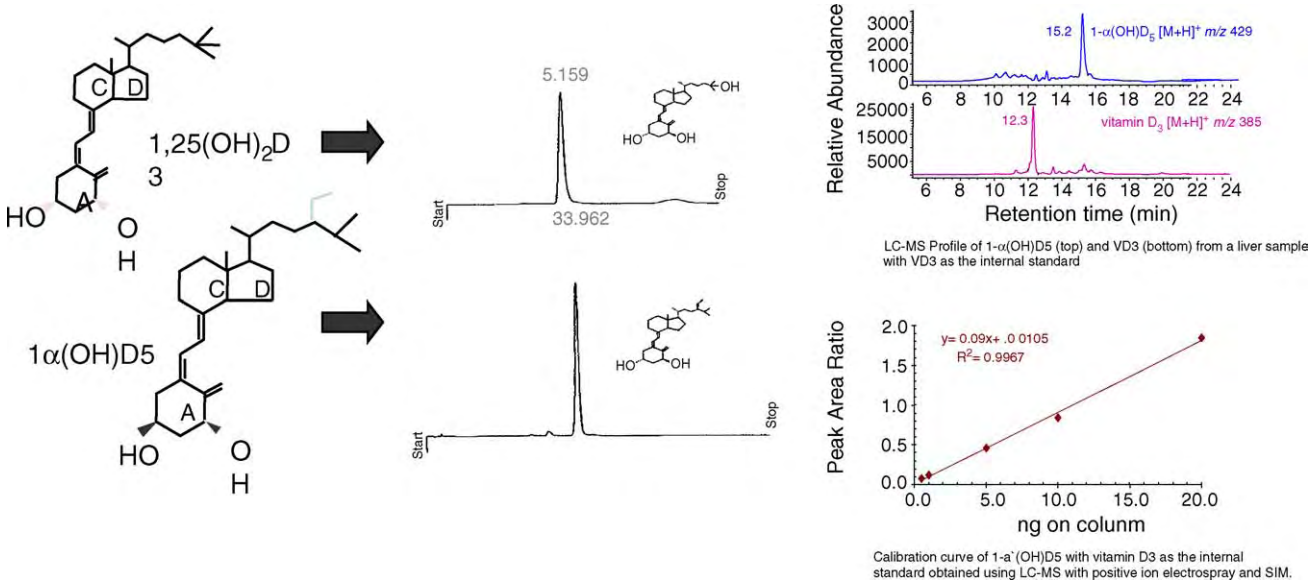


Fig. 2. Chemical Structures for 1,25-$(OH)_2D_3$ and 1α(OH)D_5 are shown with differences between the two highlighted. High-performance liquid chromatographic (HPLC) and LC–MS profiles for the two agents are shown with their respective retention times.

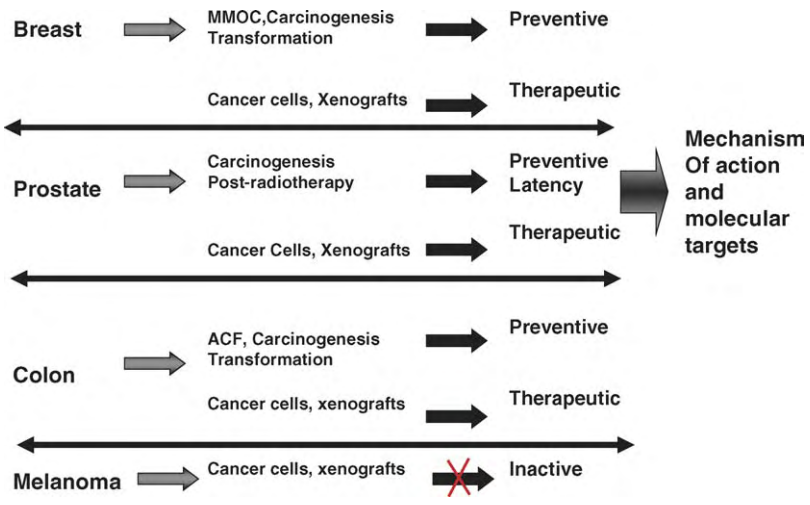


Fig. 3. Cancer models in which 1α(OH)D_5 has been tested and its proposed actions.

4. 1α(OH)D_5 in chemically-induced mammary carcinogenesis

The chemopreventive nature of 1α(OH)D_5 has been evaluated in vivo using the *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary carcinogenesis model. For this, Sprague–Dawley rats were administered 1α(OH)D_5 at a dose of either 0, 25 or 50 μg/kg diet beginning 2 weeks before carcinogen treatment. Animals received an intravenous injection of MNU (50 mg/kg body weight) at 80 days of age and continued to receive dietary 1α(OH)D_5 for an additional 120 days (Fig. 4). Tumor incidence and multiplicity were determined, and plasma concentrations of calcium and phosphorus were measured. There was no evidence of toxicity in animals treated with either 1α(OH)D_5 or carcinogen. Body weights of the rats were monitored on a weekly basis from day 0 to the time of termination with no significant differences ($p > 0.05$) observed. As shown in Fig. 4, the tumor incidence was reduced from 80% in control animals to 53.3% (CI = 26.6–78.8%) and 46.6% (CI = 21.3–73.4%) in rats treated with 1α(OH)D_5 at 25 and 50 μg/kg diet, respectively. The tumor multiplicity was reduced from 1.6 tumors per rat to 1.2 and 0.8, respectively. There was no statistically significant increase in plasma calcium or phosphorus concentration at either dose level. These results were consistent with the MMOC results previously described [22].

In a subsequent study, the stage specific effects of 1α(OH)D_5 on either the initiation or promotion during carcinogenesis were examined using the 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis design. Fifty-day old Sprague–Dawley rats were used. Animals were randomized by weight into four groups of 20 animals each and received 15 mg of DMBA in 1 mL of corn oil intragastrically. The groups' included: (1) a control (regular rat chow); (2) an initiation + promotion (a diet supplemented with 1α(OH)D_5 from 2 weeks prior to the carcinogen until the end of the study); (3) an initiation (diet supplemented with 1α(OH)D_5 from 2 weeks prior to the carcinogen treatment to the week after the carcinogen); (4) a promotion (1α(OH)D_5 beginning 1 week after the carcinogen treatment until the end of the study (Fig. 4)). Two additional negative control groups were also included with 10 rats per group receiving no carcinogen and either placebo or the 1α(OH)D_5 supplemented diet. As shown in Table 1, 1α(OH)D_5 (40 μg/kg diet) inhibited cancer incidence by 37.5% ($p < 0.05$) if 1α(OH)D_5 was present in food during

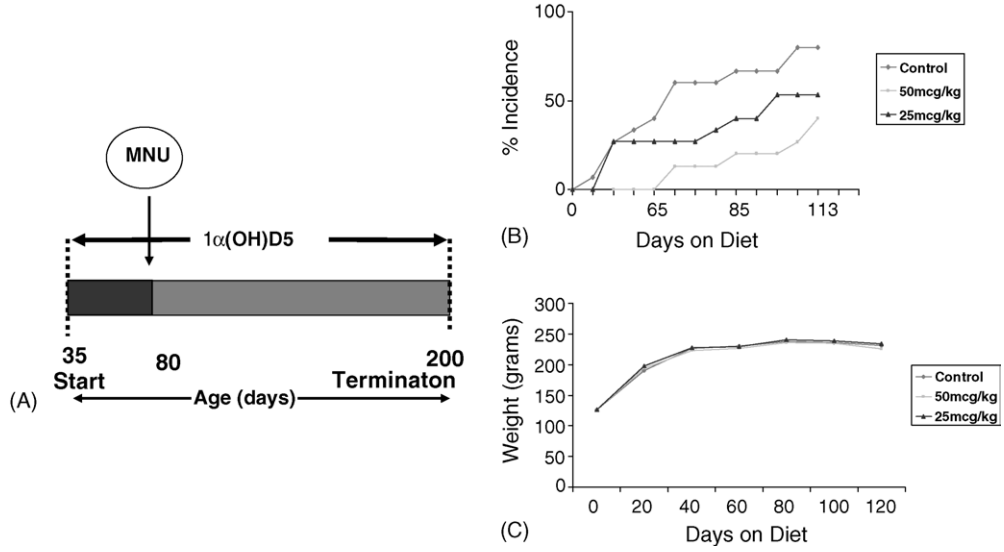


Fig. 4. (A) Experimental design used to evaluate the effects of 1α(OH)D_5 on *N*-methyl nitrosourea (MNU)-induced mammary carcinogenesis. Sprague Dawley female rats were given intraperitoneally at a dose of 50 mg of MNU/kg body weight at 80 days of age. (B) The rats were administered a basal diet (♦) or a diet supplemented with either 25 μg (▲) or 50 μg (■) of 1α(OH)D_5/kg diet starting at day one and continued throughout the study. The animals were palpated for the presence of tumors and weighed once weekly. Animals were sacrificed at 230 days old. (C). Tumor incidence was reduced from 80% in control animals (♦) to 53.3% and 46.6% in rats treated with 1α(OH)D_5 at 25 (▲)and 50 μg/kg diet (■), respectively. No statistical difference in body weights of animals was observed (Adapted from: [31]).

Table 1
Chemopreventive efficacy of 1α(OH)D_5 in 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis in rats

Treatment	*N*	Schedule (weeks)	Incidence (%)	Latency (days)	Final BW (g)
Control	20	–	17/20 (85)	43	265
D_5 (40 mg/kg)	20	−2 to end	10/20 (50)	63	254
D_5 (40 mg/kg)	20	−2 to +1	14/20 (70)	59	266
D_5 (40 mg/kg)	20	+1 to end	10/20 (50)	70	255

the promotion phase (+1 week to end). The 1α(OH)D_5-supplemented diet during the initiation phase (−2 to 1 week) did not provide any significant protection, the observed inhibition was only 12.5%. No significance in the difference of body weights or in the circulating levels of calcium or phosphorous were found between control or treatment groups. The findings show that the effects of vitamin D_5 may be mediated selectively during the promotion or progression phases of carcinogenesis. These observations further support the development of 1α(OH)D_5 as a potential chemopreventive and/or therapeutic agent in breast cancer.

5. Vitamin D and its analogs in colon cancer chemoprevention

As previously mentioned, the strongest epidemiological evidence for the protective effects of vitamin D has been suggested for colon cancer [10]. In addition, several analogs of vitamin D have been evaluated as potential colon cancer chemopreventive agents. For example, R024-5531, the hexafluoro analog of vitamin D_3, has been investigated [25] for the prevention of colon cancer. It was reported that the administration of R024-5531 for 34 weeks resulted in 40% reduction of colon tumor formation in treatment groups. Similarly, in another study Otoshi et al. [26] showed that the IP injections of 22-oxa-calcitriol suppressed the development of aberrant crypt foci in rats. Based on data, we hypothesized that 1α-hydroxyvitamin D_5 could serve as an effective non-toxic vitamin D analog for the prevention and treatment of colon cancer.

5.1. 1α(OH)D_5 in chemically-induced colonic aberrant crypt foci

A study was conducted to test the efficacy of 1α(OH)D_5 as a potential chemopreventive agent in colon carcinogenesis. Since the formation of ACF in response to carcinogen exposure is considered as one of the earliest recognizable precancerous lesions and the ACF model has been extensively used to identify modulators of colon carcinogenesis [27], we investigated the effects of 1α(OH)D_5 on the incidence and multiplicity of azoxymethane (AOM)-induced ACF in mice. CF-1 mice were treated s.c. with 10 mg/kg AOM weekly for 2 weeks. 1α(OH)D_5 was supplemented in the diet (25 μg/kg diet) beginning 7 days before the carcinogen treatment and continued until the termination of the study. Blood was collected at the time of sacrifice and calcium levels were determined. The levels of calcium for the animals were 9.51 ± 0.6 and 9.36 ± 0.4 mg/dl for control and 1α(OH)D_5, respectively. Furthermore, no differences in weight or level of activity between the groups were noted. Colons were stained with methylene blue and scored for the presence of ACF. Results show that 1α(OH)D_5 significantly ($p < 0.001$) reduced the incidence and multiplicity of ACF (29 ± 3.2 versus 1.4 ± 0.5) in animals receiving control and 1α(OH)D_5, respectively.

In a second study, we investigated whether 1α(OH)D_5 selectively inhibits AOM-induced colon ACFs during either the initiation or promotional stages of carcinogenesis. As in the previous study, CF-1 mice were treated s.c. with 10 mg/kg AOM weekly for 2 weeks and 1α(OH)D_5 was supplemented in the diet at a dose of 25 μg/kg diet. The groups included: (1) a control (regular rat chow); (2) an initiation + promotion (a diet supplemented with 1α(OH)D_5 from 2 weeks prior to the carcinogen until the end of the study [−2 weeks to end]); (3) an initiation (diet supplemented with 1α(OH)D_5 from 2 weeks prior to the carcinogen treatment to the week after the carcinogen [−2 weeks to +1 week]); (4) a promotion (1α(OH)D_5 beginning 1 week after the carcinogen treatment until the end of the study (+1 week to end) (Fig. 5). As in the previous study, colons were scored for the presence and size of ACF. Hexosaminidase, a lysosomal enzyme active in normal colonic crypts yet showing decreased activity in

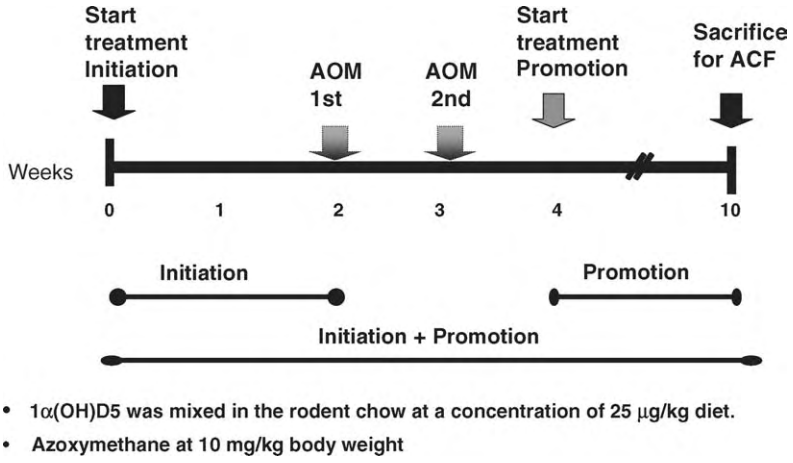
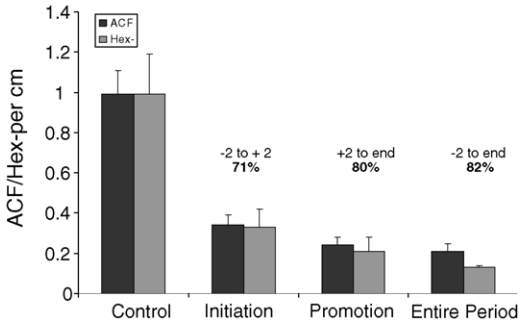


Fig. 5. Experimental design used to evaluate the effects of 1α(OH)D_5 on AOM-induced colon aberrant crypt foci in CF-1 mice. In protocol A, 1α(OH)D_5 was administered from day 0 until a day prior to first carcinogen injection. For protocol B, 1α(OH)D_5 was administered one week following the last carcinogen injection till the end of the study. In Protocol C, 1α(OH)D_5 was administered from day 0 to the end of the study.

(A)



(B)

Treatment Period	Small ACF <6 crypts	Large ACF >6 crypts
Control	8.6 + 0.81	1.0+0.7
-2 to +2 wks (Initiation)	2.6 +1.67* (70)	0.2 +0.2* (80)
+2 to End (Promotion)	2.0 +0.95* (77)	0.0 + 0* (100)
-2 to End (Entire period)	1.4+0.4* (84)	0.4 + 0.24 (60)

Fig. 6. (A) Mean number of Aberrant crypt foci and loss of hexosaminidase activity per per square cm of colon for control, initiation, promotion and entire period. Initiation group received agent starting 2 weeks before carcinogen until 2 weeks after 1st AOM injection. The promotion group received $1\alpha(OH)D_5$ (25 μg/kg diet) starting 2 weeks after the 1st AOM injection until the end of the study. A third group referred to as entire period, was administered $1\alpha(OH)D_5$ throughout the study. (B) Analysis of the size of ACF presented as small (≤6 crypts) vs. larger crypts (>6 crypts). The mean number (±S.E.) of large crypts was reduced by 80, 100 and 60% for initiation, promotion and entire period groups, respectively.

the ACF, was used as a second biomarker for colon carcinogenesis. As shown in Fig. 6, colons of animals receiving $1\alpha(OH)D_5$ had significantly less ACFs when compared to the colons of control animals. A reduction of 71, 80 and 82% was observed for animals receiving $1\alpha(OH)D_5$ during the initiation, promotion, and entire period, respectively. Loss of activity of hexosaminidase (hex-foci) was prevented by $1\alpha(OH)D_5$ in a manner comparable to that of ACF. In addition, $1\alpha(OH)D_5$ significantly reduced the number of large (>6 abnormal crypts) ACF, when compared to the control diet (80, 100 and 60%), initiation, promotion and initiation + promotion, respectively). These results are of particular significance since large ACF have been reported to better correlate with tumor incidence. Thus, these data collectively

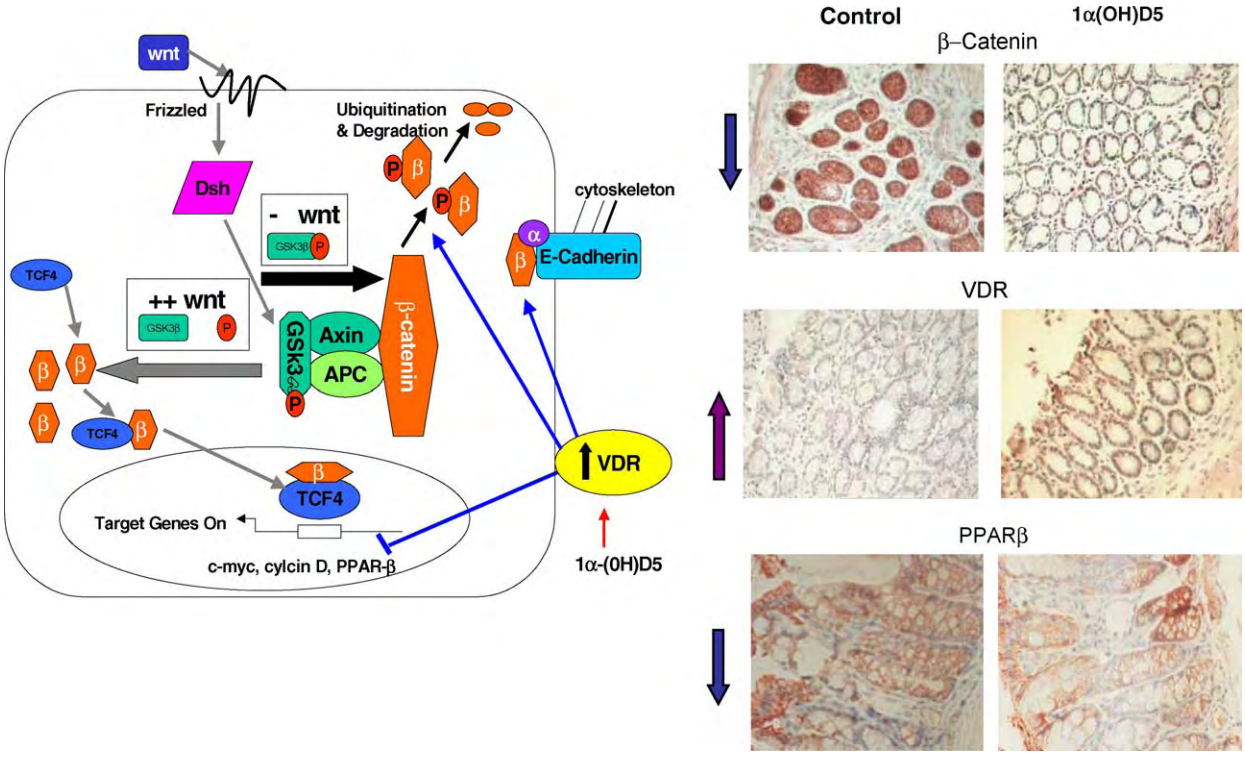


Fig. 7. Schematic diagram of the *Wnt*/beta-catenin pathway. Potential actions of $1\alpha(OH)D_5$ on the members of the pathway are highlighted. Colon sections from AOM-treated mice receiving either control or $1\alpha(OH)D_5$ supplemented diet. Nuclear β-catenin and peroxisome proliferator-activated receptor (PPAR) beta were both found to be decreased in colon sections of animals receiving $1\alpha(OH)D_5$ as compared to the colons from the control mice. In contrast, VDR levels were found to be up-regulated in animals receiving $1\alpha(OH)D_5$.

provide strong support for the potential use of $1\alpha(OH)D_5$ as a cancer chemopreventive agent.

5.2. The Wnt/β-catenin pathway as potential target for $1\alpha(OH)D_5$ in the colon

Beta-catenin was originally identified as a protein that interacts with the cytoplasmic domain of E-cadherin, important for cell-cell adherence junctions [28]. Later studies revealed that beta-catenin plays a central role in the *Wnt* signaling pathway (Fig. 7). In colon cancer, deregulation of the *Wnt* pathway is a well-defined early event [29]. Specifically, mutations in the β-catenin gene prevent the protein from being phosphorylated on specific serine and threonine residues in the N-terminal region, thereby impeding its degradation. The resulting accumulation of β-catenin in the cell cytoplasm and the subsequent translocation to the nucleus leads to the formation of active β-catenin/TCF complexes and constitutive target proliferative genes and oncogenes. Recent evidence suggests the cancer chemopreventive agents may act via reversing oncogenic APC/β-catenin/Lef/Tcf signals. In a recent study, Palmer et al. [30] investigated whether 1,25-$(OH)_2D_3$ and several vitamin D analogs had any effects on the β-catenin signaling pathway. Using two sub-populations of the SW480 cell line (SW480-Adh VDR+ and SW480-R VDR−), 1,25-$(OH)_2D_3$ was shown to inhibit the transcriptional activity of β-catenin, by increasing the amount of VDR bound to β-catenin; inhibit the expression of β-catenin-TCF-4 responsive genes (e.g., c-myc, CD44); inducing E-cadherin. In view of these studies, we investigated whether administration of $1\alpha(OH)D_5$ had any effects on the *wnt* signaling proteins.

In order to investigate whether administration of $1\alpha(OH)D_5$ modulated members of the *wnt* signaling pathway, formalin-fixed, paraffin-embedded colon sections of AOM-treated animals receiving control or $1\alpha(OH)D_5$ supplemented diets were processed for immunohistochemistry studies. Samples were incubated with β-catenin, VDR (Neomarkers, Freemont, CA) and peroxisome proliferator-activated receptor β (PPAR-β) (Santa Cruz, Santa Cruz, CA) for 2 h at room temperature. Immunoperoxidase reaction was performed using the DAKO LSAB2 System kit (DAKO Corporation, Carpinteria, CA). Briefly, the biotinylated link IgG was applied for 10 min, followed by incubation of horseradish peroxidase (HRP) linked streptavidin. After washing the sections with PBS, AEC substrate–chromogen solution was applied. The sections were then counterstained with hemotoxylin and examined under the microscope. As shown in Fig. 7, administration of $1\alpha(OH)D_5$ inhibited expression of nuclear β-catenin and PPAR-β, a β-catenin-TCF-4 responsive gene and induced expression of VDR. These results show that $1\alpha(OH)D_5$ may be mediating its effects in part by inhibiting the β-catenin signaling pathway, which has an anti-tumor effect in vivo. These results point to the key role of VDR expression in colon carcinogenesis and support further study of $1\alpha(OH)D_5$ for the treatment of colon cancer.

6. Summary

Studies were conducted to evaluate the efficacy of $1\alpha(OH)D_5$ as a potential chemopreventive analog of vitamin D in experimental mammary and colon carcinogenesis. Results show that $1\alpha(OH)D_5$ was effective in inhibiting the development of preneoplastic lesions in the mammary glands. Furthermore, in MNU- and DMBA-induced mammary carcinogenesis models, $1\alpha(OH)D_5$ reduced the incidence of mammary tumors as well as the multiplicity and the effects were selective during the promotional phase of carcinogenesis. In the colon, administration of $1\alpha(OH)D_5$ resulted in inhibition of number of ACF as well as reduction in size of ACF in AOM-treated animals. Consistently, in all in vivo studies, no signs of toxicity at efficacious doses have been noted.

Mechanistic studies on $1\alpha(OH)D_5$, using several breast and colon cancerous cells, have cumulatively demonstrated that $1\alpha(OH)D_5$ mediates its effects via VDR. Furthermore, administration of $1\alpha(OH)D_5$ has been shown to induce a G1 cell cycle arrest in several cancer cell lines [24]. In the colon, it appears that $1\alpha(OH)D_5$ may be mediating its effects in part via inhibition of the oncogenic β-catenin pathway. Thus, these results suggest that $1\alpha(OH)D_5$ may be functioning via multiple signaling pathways to inhibit cancer development. Collectively, both in vitro and in vivo studies have demonstrated the potential use of $1\alpha(OH)D_5$ as a possible chemopreventive agent in breast and colon carcinogenesis.

Acknowledgements

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Expression of Vitamin D Receptor and 25-Hydroxyvitamin D3-1α-Hydroxylase in Normal and Malignant Human Colon

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Abstract

Considerable evidence exists to support the use of vitamin D to prevent and/or treat colorectal cancer. However, the routine use of bioactive vitamin D, 1,25-dihydroxyvitamin D3, is limited by the side effect of toxic hypercalcemia. Recent studies, however, suggest that colonic epithelial cells express 25-hydroxyvitamin D3-1α-hydroxylase, an enzyme that converts nontoxic pro-vitamin D, 25-hydroxycholecalciferol [25(OH)D3], to its bioactive form. Yet, nothing is known as to the cellular expression of 1α-hydroxylase and the vitamin D receptor (VDR) in the earliest histopathologic structures associated with malignant transformation such as aberrant crypt foci (ACF) and polyps [addressing the possibility of using nontoxic 25(OH)D3 for chemoprevention], nor is anything known as to the expression of these proteins in colorectal cancer as a function of tumor cell differentiation or metastasis [relevant to using 25(OH)D3 for chemotherapy]. In this study, we show that 1α-hydroxylase is present at equal high levels in normal colonic epithelium as in ACFs, polyps, and colorectal cancer irrespective of tumor cell differentiation. In contrast, VDR levels were low in normal colonic epithelial cells; were increased in ACFs, polyps, and well-differentiated tumor cells; and then declined as a function of tumor cell de-differentiation. Both 1α-hydroxylase and VDR levels were negligible in tumor cells metastasizing to regional lymph nodes. Overall, these data support using 25(OH)D3 for colorectal cancer chemoprevention but suggest that pro-vitamin D is less likely to be useful for colorectal cancer chemotherapy. (Cancer Epidemiol Biomarkers Prev 2005;14(10):2370–6)

Introduction

Vitamin D's ability to prevent colorectal cancer has been suspected for over a quarter of a century. One of the earliest studies supporting this link came from the observation that there was an inverse relationship between mean solar radiation and age-adjusted colorectal cancer death rates (1). Since that time, a number of epidemiologic studies have suggested a link between vitamin D and/or calcium levels and the incidence of human colorectal cancer formation (reviewed in ref. 2). The validity of these epidemiologic observations was significantly enhanced by the results of the Calcium Polyp Prevention Study, a double-blind placebo-controlled study that showed a decreased recurrence rate of colorectal adenomas in patients receiving calcium carbonate (3, 4). Patients receiving the greatest benefit from calcium supplementation were those with decreased serum levels of 25-hydroxycholecalciferol [25(OH)D3; ref. 3], highlighting the importance of vitamin D in inhibiting colorectal cancer formation.

Although vitamin D status in humans is typically assessed by measuring serum 25(OH)D3 levels, this nontoxic pro-vitamin requires hydroxylation at carbon 1 by 25-hydroxyvitamin D3-1α-hydroxylase to generate bioactive 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]. Whereas the primary function of 1,25(OH)2D3 is to regulate calcium absorption and maintain mineral homeostasis, upon binding to its cognate vitamin D receptor (VDR), 1,25(OH)2D3 also decreases the proliferation and enhances the differentiation of colorectal cancer cells (5) as well as alters the transcription of a large number of genes involved in inhibiting carcinogenesis (6, 7).

Unfortunately, 1,25(OH)2D3 has a narrow therapeutic index, with its propensity for causing toxic hypercalcemia precluding its routine use in otherwise healthy patients. Recently, however, a number of investigators have suggested that 1α-hydroxylase is present in normal (8, 9) and malignant (10, 11) epithelial cells lining the adult human colon, suggesting that nontoxic 25(OH)D3 might represent an efficacious treatment modality for treating patients with known colorectal polyps or cancer (i.e., chemotherapy), or preventing these lesions from even forming in the first place (i.e., chemoprevention). Yet, the studies done to date have not systematically evaluated VDR and 1α-hydroxylase expression in colon cancer as a function of tumor cell differentiation or metastasis, critical to assessing 25(OH)D3's potential as a chemotherapeutic agent, nor have they evaluated the expression profile of these proteins in early neoplastic lesions such as aberrant crypt foci (ACF), critical to assessing 25(OH)D3's potential as a chemopreventive agent.

To assess 25(OH)D3's potential for colorectal cancer chemoprevention and/or chemotherapy, we herein report on cellular VDR and 1α-hydroxylase protein expression in human ACFs, polyps, and colorectal cancer's of defined differentiation along with associated lymph node metastases. To do this, we used quantitative immunohistochemistry to precisely determine VDR and 1α-hydroxylase expression in formalin-fixed surgically resected tissues at the cellular level. We herein show that 1α-hydroxylase levels are consistently high in all nonmetastatic tissues, irrespective of histology. In contrast, we show that VDR levels are increased in ACFs and well-differentiated tumors but progressively diminish with colorectal cancer de-differentiation. Lastly, we show that both 1α-hydroxylase and VDR expression is low to negligible in metastatic tissues, regardless of the histopathologic stage assigned to either the primary tumor or tumor contained

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within the metastasis. Overall, these findings suggest that 25(OH)D3 has potential for use in colorectal cancer chemoprevention but may be less efficacious for colorectal cancer chemotherapy.

Materials and Methods

Materials. Anti-VDR antibody was from Abcam (Cambridge, MA). Sheep Anti-murine 25-hydroxyvitamin D3-1α-hydroxylase antibody was from The Binding Site (San Diego, CA). Goat anti-sheep horseradish peroxidase (HRP)–labeled antibody, goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TBST wash buffer, target retrieval solution, protein block serum, antibody diluent, EnVision+ HRP, (DAB)-rabbit system, 3,3′ -diaminobenzidine (DAB) chromogen, and automated hematoxylin were all from DAKO (Carpinteria, CA). Auto/iodine, Redusol, and Permount and polyvinylidene difluoride membranes were from Fisher Scientific (Pittsburgh, PA). Mammalian protease inhibitor cocktail was purchased from Sigma (St. Louis, MO). Enhanced Chemiluminescence plus Western Blotting Detection System was from Amersham (Piscataway, NJ); 30% acrylamide/Bis solution and Precision Plus protein standards were from Bio-Rad Laboratories, Inc. (Richmond, CA.); and bicinchoninic acid protein assay kit was from Pierce (Rockford, IL). Calcitriol (1α, 25-dihydroxyvitamin D3) was purchased from BIOMOL International, LP (Plymouth Meeting, PA).

Tumor Specimens and Histologic Grading. Colon cancers were randomly selected from the University of Illinois at Chicago Gastrointestinal Tumor Bank. The University of Illinois at Chicago and Veterans Administration Institutional Review Boards approved use of these tissues for this study under the stipulation that no linked clinical data could be used as a part of their evaluation and analysis.

Differentiation was assessed as previously described (12-14). Briefly, well-differentiated tumors were defined by the presence of well-formed glands containing malignant columnar cells displaying small regular nuclei. The complete absence of gland formation, or the presence of bizarrely shaped glands, identified poorly differentiated tumors. Moderately differentiated tumors possessed well-formed glands, but the cells were less columnar or frankly cuboidal, with reduced cell polarity and more dysplastic nuclei than those observed in well-differentiated tumors.

Western Analysis. Confluent cells were rinsed in PBS and lysed in radioimmunoprecipitation assay buffer containing a 1:20 dilution of mammalian protease inhibitor cocktail. Protein concentrations were determined using the bicinchoninic acid protein assay kit as described by manufacturer. In all instances, 20 μg of protein were loaded and electrophoresed across a 10% polyacrylamide gel under denaturing and reducing conditions. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibody for 2 hours at the following concentrations: VDR, 1:1,750; α(OH), 1:150; and actin, 1:100 followed by two sequential 10-minute washes with TBST. VDR and actin immunoreactive bands were visualized using a HRP-conjugated goat anti-rabbit IgG, whereas α(OH) was visualized using a HRP-conjugated rabbit anti-goat IgG and the Enhanced Chemiluminescence Plus detection system.

Quantitative Immunohistochemistry. Tissues were sectioned (4 μm thick) using a Sakura Accu-Cut SRM 200 Rotary Microtome (Torrance, CA) and processed for antigen retrieval in the following manner. Sections were rehydrated in graded alcohol and a running water bath, placed in auto/iodine for 1 minute, rinsed in Tris-buffered saline Tween 20 (TBST) buffer, rinsed twice for 2 minutes in Redusol, and once again in TBST buffer. The slides were then placed in Target Retrieval Solution at 100°C for 20 minutes, allowed to cool to room temperature, and rinsed once again in TBST.

Immunohistochemistry was done on a DAKO Autostainer Universal Staining System using a two-step indirect immunoperoxidase technique. Briefly, tissues were incubated in a 3% H_2O_2 solution to quench endogenous peroxidase activity, rinsed with TBST, blocked with protein block serum for 30 minutes, and rinsed in TBST. VDR primary antibody was applied (1:1,750, as determined by antibody titration) for 1 hour at room temperature before rinsing with TBST. Next, labeled polymer rabbit HRP was added for 30 minutes, rinsed thoroughly with TBST followed by incubation with DAB^+ chromogen for 8 minutes, and then counterstained for 2 minutes with hematoxylin.

1α-Hydroxylase primary antibody was applied (1:150, as determined by antibody titration) for 45 minutes at room temperature before rinsing with TBST. Next, rabbit anti-sheep HRP-labeled antibody was applied (1:100) for 45 minutes. Slides were rinsed with TBST followed by DAB chromogen incubation for 5 minutes and counterstained for 2 minutes with hematoxylin. All tissues were then dehydrated in graded alcohol and xylene and cover-slipped using Permount. For all specimens, control tissues were processed identically and at the same time, except that they were not exposed to primary antibody. Thus, all differences between the experimental tissue and the control tissue are ultimately due to DAB identification of the relevant protein.

Chromogen abundance was quantified by quantitative immunohistochemistry as previously described (15, 16). Briefly, images were acquired using a Diagnostic Instruments SPOT RT Digital Scanning Camera (Sterling Heights, MI) attached to a Nikon E600 microscope (Stamford, CT), and image files saved in tagged-image file format. The amount of chromogen per pixel was determined by subtracting the mathematical energy (E_M) of the control slide (i.e., not exposed to primary antibody) from that in the homologous region of the experimental slide (i.e., exposed to primary antibody). Chromogen quantity (E_M) is expressed as energy units per pixels (eu/pix).

Standards and Statistics. In all instances, immunohistochemical quality control was achieved as follows. Because all runs were done using a computer-controlled DAKO Autostainer, conditions were identical at all times. Additionally, a section from a single tumor was included in all runs to control for experimental variability. The amount of chromogen as determined by quantitative immunohistochemistry revealed run-to-run variability of <5%. Data obtained by quantitative immunohistochemistry was evaluated by using the online statistical calculator provided by the College of Saint Benedict/St. John's University (http://www.physics.csbsju.edu/) using the statistical test as identified in the text, with $P < 0.05$ considered significant.

Results

Few studies exist examining 1α-hydroxylase or VDR protein expression in the colon, whereas no studies have systematically evaluated their expression as a function of tumor cell differentiation. To evaluate the expression of these proteins, we used commercially available polyclonal antibodies: the VDR antibody recognizes amino acids 395 to 413 of the human protein, whereas the 1α-hydroxylase antibody recognizes the murine protein (specific epitope not defined by the manufacturer). To confirm the specificity of these antibodies, we first evaluated them by Western blot analysis against whole protein lysates obtained from NCM460 cells, a nonmalignant human colon epithelial cell line, and Caco-2 and HT-29 cells, human

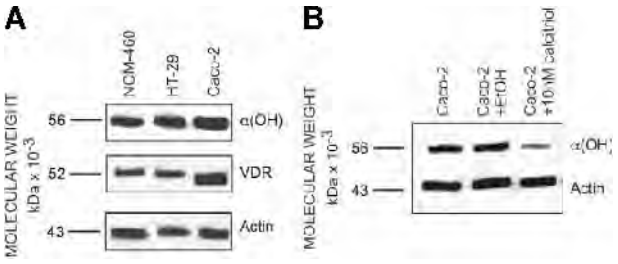

Figure 1. Western blot analysis showing 1α-hydroxylase [α(OH)] and VDR protein expression in human colonic cell lines. **A.** Protein lysates from malignant cell lines (Caco-2 and HT-29) and a nonmalignant cell line (NCM-460) were used to ensure the specificity of 1α-hydroxylase and VDR antibodies, with actin expression shown as a control. **B.** Additional evidence of specificity for the 1α-hydroxylase antibody as shown by the ability of a 48-hour incubation with calcitrol [but not ethanol (*EtOH*) vehicle] to down-regulate the appropriate band.

colon cancer cell lines. The antibody for 1α-hydroxylase identified a single band at ~56 kDa, whereas that for VDR identified a band at ~52 kDa (Fig. 1A). We further confirmed the specificity of the 1α-hydroxylase antibody, because the specific epitope was not defined by the manufacturer. Because it has been previously shown that 1,25(OH)2D3 suppresses 1α-hydroxylase expression in Caco-2 cells (8), we assessed the response of these cells to this secosteroid. In all instances, 50,000 cells were plated in defined medium in 12-well plates and cultured under standard conditions for 24 hours. Cells were then cultured for another 48 hours in serum-free medium alone, serum-free medium containing 0.1% ethanol (v/v), or serum-free medium supplemented with 10 nmol/L calcitrol in 0.1% ethanol (v/v). Caco-2 cells alone or exposed to ethanol vehicle showed strong evidence of 1α-hydroxylase expression by Western analysis, but this band was specifically and significantly down-regulated after exposure to 10 nmol/L 1,25(OH)2D3 for 48 hours (Fig. 1B) thus confirming the specificity of this commercially available antibody.

We next used these antibodies to evaluate 1α-hydroxylase or VDR expression in resected human colon cancers. To do this, we randomly selected 10 colorectal cancer from the University of Illinois at Chicago Gastrointestinal Tumor Bank. Tumor blocks selected for evaluation were those that contained the tumor margin thereby allowing us to evaluate 1α-hydroxylase or VDR protein expression in normal and malignant colonic epithelial cells from the same patient (Figs. 2 and 3). Because colorectal cancer is heterogeneously differentiated (12, 17, 18), we also could assess the expression of these proteins as a function of tumor cell differentiation, again with all evaluation done in the same patient. Overall, the 10 colorectal cancers studied contained 53 separate and distinct regions of well-differentiated tumor, 66 that were moderately differentiated, and 32 that were poorly differentiated.

Overall, 1α-hydroxylase expression seemed consistently strong in normal and malignant colonic epithelial cells, irrespective of tumor cell differentiation (Fig. 2A-D). In contrast, minimal VDR expression was observed in normal colonic epithelial cells, with the expression localized predominantly in the nucleus (Fig. 3A). With malignant transformation, total cellular VDR expression increased markedly in well-differentiated tumors (Fig. 3B) but then decreased with tumor cell de-differentiation (Fig. 3C-D).

To more accurately quantify 1α-hydroxylase and VDR expression in these tissues, we used our novel technique of

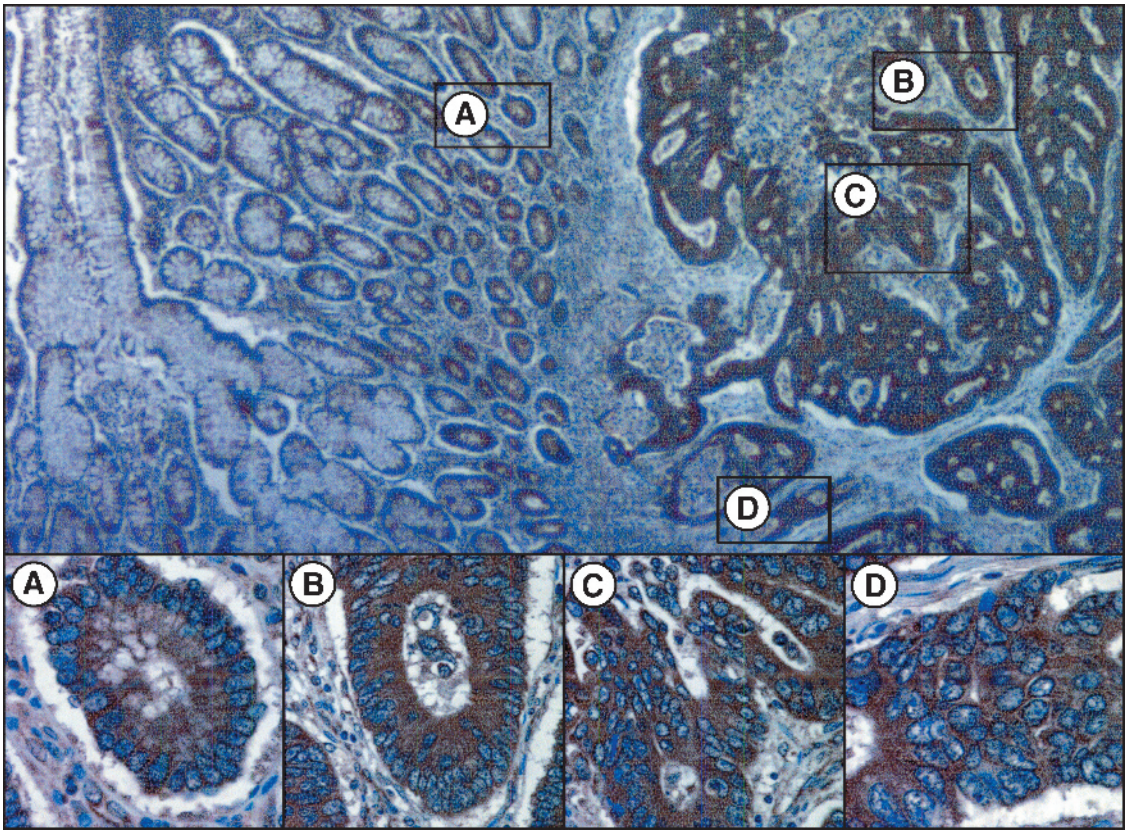

Figure 2. Expression of 1α-hydroxylase in human colon cancer and adjacent nonmalignant tissue. Similar nonnuclear levels of 1α-hydroxylase expression is noted in normal colonic epithelial cells (**A**) as in well-differentiated tumor cells (**B**), moderately differentiated tumor cells (**C**), and poorly differentiated tumor cells (**D**). Magnification, ×40 and ×1,000 (*inset*).

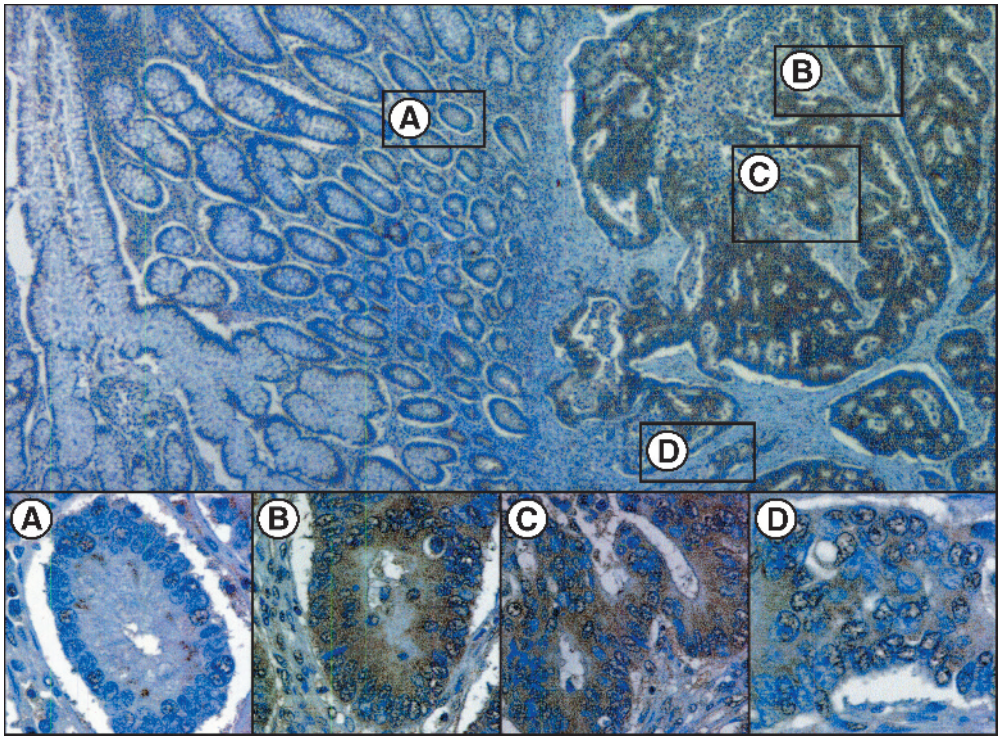

Figure 3. Expression of VDR in human colon cancer and adjacent nonmalignant tissue. VDR is expressed at low levels in normal colonic epithelial cells, with the majority of expression in the nucleus (**A**). In contrast, VDR expression is increased and predominantly cytoplasmic in well-differentiated tumor cells (**B**). However, with increasing de-differentiation, significantly less VDR expression is appreciated in moderately differentiated tumor cells (**C**) and in poorly differentiated tumor cells (**D**). Magnification, ×40 and ×1,000 (*inset*).

quantitative immunohistochemistry (15, 16), which we have shown quantifies chromogen in a manner that accurately reflects the actual amount of protein present (16). Consistent with our empirical observations as reviewed above, the amount of chromogen due to 1α-hydroxylase was of similar high levels in normal colonic epithelial cells as in well-differentiated and moderately differentiated colorectal cancer (Fig. 4). In contrast, 1α-hydroxylase levels were significantly decreased in poorly differentiated tumor cells and in metastases to regional lymph nodes (Fig. 4; $P < 0.05$, ANOVA). We also studied three tubular, three villous, and three tubulovillous adenomas, neoplastic lesions that can progress to colorectal cancer. These adenomas, irrespective of histopathology, all expressed similar levels of 1α-hydroxylase as observed in normal colonocytes and well-differentiated and moderately differentiated colorectal cancer (Fig. 4). In contrast, VDR expression was ~5-fold higher in polyps and ~12-fold higher in well-differentiated colorectal cancer than in normal colonic epithelial cells (Fig. 4; $P < 0.05$, ANOVA). However, this elevation decreased in de-differentiated colorectal cancer such that poorly differentiated tumor cells expressed VDR levels similar to what we observed in polyps, an amount that was significantly less than observed in well-differentiated tumor cells ($P < 0.05$, ANOVA).

The earliest histopathologic lesion associated with colorectal cancer malignant transformation is the ACF (19, 20). These lesions are not typically seen during routine colonoscopy but can be visualized with high-magnification endoscopes when suitable contrast stains, such as methylene blue, are used (ref. 21; Fig. 5A). Because screening colonoscopy at the University of Illinois at Chicago is routinely done using magnification chromocolonoscopy, our Gastrointestinal Tumor Bank has a large collection of human ACFs for study. We therefore randomly selected five ACF for immunohistochemical evaluation. Whereas 1α-hydroxylase expression levels were similar to what we observed in normal colonic epithelia and in polyps (Figs. 4 and 5B), the amount of VDR detected was the same as observed in polyps (Figs. 4 and 5C). Overall, then, these data suggest that 1α-hydroxylase and VDR expression levels in colorectal cancer precursor lesions such as ACFs and polyps may well allow for provitamin D drugs such as 25(OH)D3 to be used for colorectal cancer chemoprevention.

Importantly, there has been no significant improvement in the survival of patients with solid tumors that metastasize, including those affecting the colon, since the 1950s (22). Hence, we next studied all resected lymph nodes from additional five patients with known lymph node metastases. Overall, 24 separate lymph nodes (range, 2-9 per patient) were evaluated, of which 12 contained metastatic colorectal cancer. Of these, five contained metastatic tumor deposits that histologically were well differentiated (Fig. 6). We quantified the amount of VDR chromogen in well-differentiated tumor cells contained within the primary cancer and in the five well-differentiated lymph node metastases. High levels of VDR-specific chromogen were detected in well-differentiated tumor cells located within the primary cancer (402 ± 28 eu/pix, mean ± SE), whereas little to no VDR-specific chromogen was detected in well-differentiated tumor deposits metastatic to regional lymph nodes (34 ± 5 eu/pix; $P < 0.05$, unpaired t test; Fig. 7).

Because VDR acts by translocating to the nucleus after binding ligand, we used our technique of quantitative immunohistochemistry to assess the relative amounts of VDR expressed in nuclear and nonnuclear (i.e., cytoplasmic) regions (Fig. 8). Intriguingly, the ratio of nuclear to cytoplasmic VDR exceeded 4 in normal colonic epithelial cells, indicating that most of this protein was in the nucleus (Fig. 8, *inset*; Fig. 3A). However, this ratio dropped to <1.0 for ACFs, the earliest lesion associated with colorectal cancer malignant transformation.

This ratio remained between 0.9 and 0.4 for all histopathologic types evaluated except for poorly differentiated colorectal cancer and tumors metastatic to regional lymph nodes (ratio, <0.2; Fig. 8, *inset*; Fig. 3D). Thus, these data suggests that an ever-declining percentage of VDR translocates, or is able to translocate, to the nucleus of colonic epithelial cells that are not histologically normal. In concert with our observation that 1α-hydroxylase and VDR are essentially not present in metastatic cells, these data suggest that pro-vitamin D analogues such as 25(OH)D3 may not be efficacious for colorectal cancer chemotherapy.

Discussion

Vitamin D deficiency is well established as a risk factor for colorectal cancer (reviewed in ref. 2). Upon binding to the VDR, 1,25(OH)2D3 decreases the proliferation of a variety of human colon cancer cell lines (23, 24) as well as of cells within resected human rectal mucosa (25). Moreover, VDR-null mice exhibit decreased markers of cellular proliferation and increased levels of markers reflecting DNA oxidative stress (26), suggesting that the vitamin D-receptor complex acts by attenuating oxidative DNA damage and preventing malignant transformation from occurring. Additionally, once malignant transformation has occurred, 1,25(OH)2D3 induces apoptosis (27, 28) as well as has pro-differentiating effects (27) on various colorectal cancer cell lines. Thus, vitamin D may have colorectal cancer chemotherapeutic in addition to chemopreventive effects.

Despite the evidence for increasing hypovitaminosis D in the general population (29-31), widespread use of 1,25(OH)2D3 is not likely to occur given its narrow therapeutic range and side effect profile, even in individuals with known colorectal cancer. However, a role for nontoxic 25(OH)D3 in colorectal cancer chemoprevention and chemotherapy might be reasonable if 1α-hydroxylase is present, along with the VDR, in relevant locations so as to allow for local generation of bioactive vitamin D. Hence, the purpose of the current study was to systematically assess 1α-hydroxylase and VDR cellular expression in normal and neoplastic colonic epithelium.

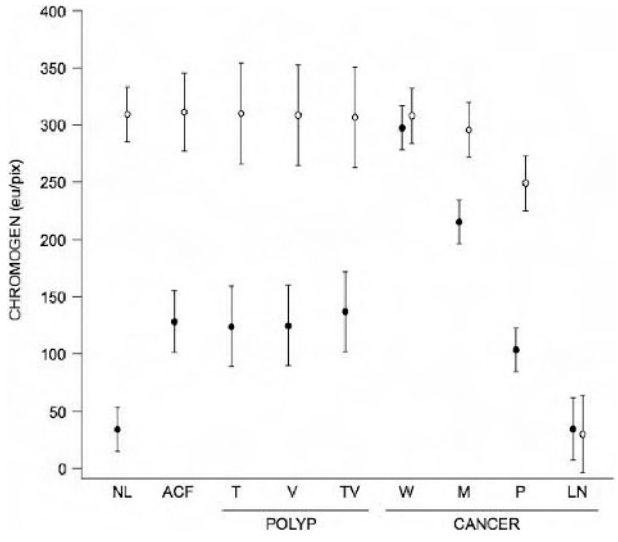

Figure 4. Amount of 1α-hydroxylase (○) and VDR (●) present in normal colonic epithelium, ACF, polyps, colon cancers of defined differentiation, and tumors metastasizing to regional lymph nodes. In all instances, immunohistochemistry was done and chromogen amount quantified as described in Materials and Methods. *Points,* means; *bars,* 95% confidence intervals. The amount of 1α-hydroxylase in lymph nodes is significantly different from the amount present in all other tissues (ANOVA, $P < 0.01$). The amount of VDR present in ACFs and polyps are similar and significantly different from that expressed by normal colonic epithelial cells, as well as in well-differentiated and moderately differentiated cancers and metastases to lymph nodes (ANOVA, $P < 0.01$). Abbreviations: NL, normal colonic epithelium; T, tubulovillous, V, villous, TV, tubulovillous; W, well differentiated; M, moderately differentiated; P, poorly differentiated; LN, lymph node.

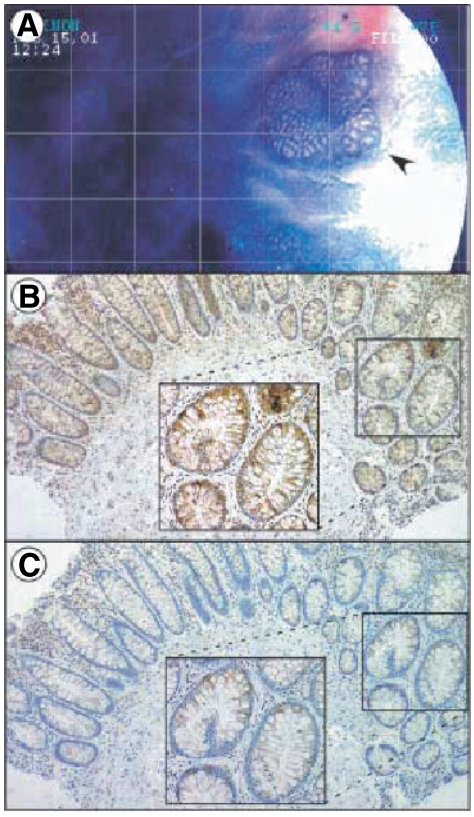

Figure 5. Expression of 1α-hydroxylase and VDR in a human ACF. **A.** ACF detected during magnification chromocolonoscopy (*arrowhead*). **B.** 1α-Hydroxylase expression in ACF (*boxed region*) with surrounding normal epithelium. Magnification, ×100. **C.** VDR expression in ACF (*boxed region*) with surrounding normal epithelium. Magnification, ×100. *Insets,* greater cellular detail provided. Magnification, ×400.

Few studies have systematically evaluated both 1α-hydroxylase and VDR expression in normal, premalignant, and malignant tissues. It has been suggested that 1α-hydroxylase expression is higher in colorectal cancer compared with adjacent normal tissues at both the mRNA (11) and protein (32) levels. In contrast, whereas some have reported increased 1α-hydroxylase expression in well-differentiated colorectal cancer compared with poorly differentiated tumors (33), others have reported the converse (10). We are aware of but two studies looking at both 1α-hydroxylase and VDR expression in colorectal cancer as a function of tumor differentiation (34, 35). In these, ''low-grade'' tumors expressed increased amounts of 1α-hydroxylase and VDR mRNA and/or protein compared with adjacent normal mucosa. These investigators reported that 1α-hydroxylase and VDR mRNA and/or protein expression then decreased as tumors de-differentiated (34, 35). Yet, these studies evaluated whole tumors and did not study 1α-hydroxylase/VDR expression at the cellular level.

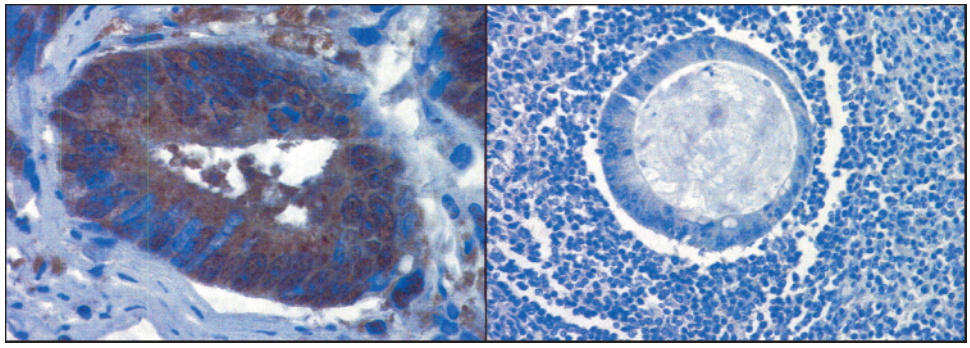

Figure 6. Expression of VDR in primary tumor (*left*) and associated lymph node metastasis (*right*). Note that tumor cells in both the primary and metastatic lesion are well differentiated but that VDR can only be detected immunohistochemically in the primary tumor. Tissues were obtained from the University of Illinois at Chicago Gastrointestinal Tumor Bank and processed as described in Materials and Methods.

Clinically, the most important implication of tumor differentiation relates to the fact that it predicts the development of metastases for most solid tumors (36, 37). In contrast, colon cancers are unusual and differ from most other solid tumors, including even rectal cancers (17), insofar they tend to be heterogeneously differentiated (12, 18). Thus, assessing an entire colon cancer's ''differentiation,'' as is usually done by clinical pathologists when assessing tumor ''grade,'' does not provide information useful for predicting a patient's outcome or survival. However, when the differentiation of specific cell populations within a colon cancer is considered, this variable does yield prognostic information (38, 39). For example, individual tumors containing well-differentiated cells at the leading edge (39), or at the point of budding (38), do better even in the face of local lymph node invasion than tumors not associated with local metastases but which are comprised of less well differentiated cells. Thus, differentiation and the factors regulating tumor cell appearance are as important in colon cancer as for other solid tumors, providing that this analysis be done at the level of the individual tumor cell. In this study, we make use of our powerful technique of quantitative immunohistochemistry (15, 16) to show for the first time the degree to which both 1α-hydroxylase and VDR are expressed at the cellular level in colonic epithelia as a function of histology. Whereas 1α-hydroxylase is expressed in uniformly high levels in normal and neoplastic colonic epithelial cells, VDR expression is low in normal colonic epithelia, is increased in better differentiated tumor cells, and decreases in poorly differentiated tumor cells, within any particular colorectal cancer. Most importantly, we show that the expression of both 1α-hydroxylase and VDR is decreased in lymph node metastases, irrespective of the degree to which the tumor cells are differentiated in the metastatic lesion.

Our technique for quantitative immunohistochemistry also permitted us to determine the amount of VDR chromogen present in nuclear and cytoplasmic regions of each cell. In so doing, we could calculate the relative amount present in the nucleus compared with the cytoplasm, a marker of VDR nuclear translocation and thus of ''activity.'' Nuclear-to-cytoplasmic VDR ratios drop dramatically in even ACFs and decline further thereafter in neoplastic lesions as a function of histology. Thus, these findings indicate that, despite uniformly high levels of 1α-hydroxylase, pro-vitamin D [i.e., 25(OH)D3] may be less likely to be of value for colorectal cancer

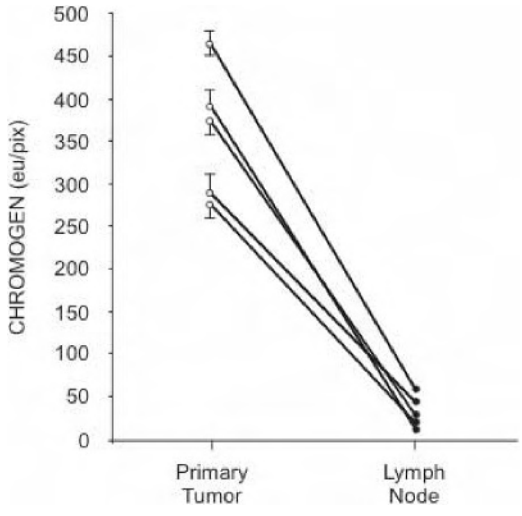

Figure 7. Amount of VDR chromogen present in primary tumors (○) and associated metastases to regional lymph nodes (●). Five tumors with known lymph node metastases were randomly selected from the University of Illinois at Chicago Gastrointestinal Tumor Bank. All surgically resected lymph nodes were evaluated for evidence of tumor infiltration. Lymph nodes containing well-differentiated tumor ($n = 5$) were processed immunohistochemically for VDR expression at the same time as the primary lesion, and chromogen quantified as described in Materials and Methods. Chromogen quantity for well-differentiated regions within the primary tumors and for the well-differentiated tumor nest within the relevant lymph node ($P < 0.05$, paired t test). *Points,* means; *bars,* ±SE.

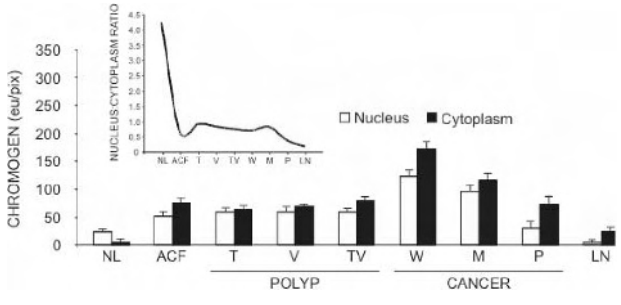

Figure 8. Nuclear versus cytoplasmic VDR expression in normal colonic epithelium, ACF, polyps, colon cancers of defined differentiation, and tumors metastasizing to regional lymph nodes. In all instances, immunohistochemistry was performed and chromogen quantified as described in Materials and Methods. *Columns,* means; *bars,* ±SE. *Inset,* nuclear to cytoplasmic ratio of VDR expression as determined by quantitative immunohistochemistry. Abbreviations: NL, normal colonic epithelium; T, tubulovillous; V, villous; TV, tubulovillous; W, well differentiated; M, moderately differentiated; P, poorly differentiated; LN, lymph node.

chemotherapy. Although the significance of this finding is not clear, it is possible, given the observation that VDR polymorphisms can result in nonfunctional protein (40, 41) and that tumor de-differentiation might be associated with increasing mutation of the *VDR* gene. If so, this would be similar to what has been observed for the gene for the colon cancer morphogen, gastrin-releasing peptide receptor (13).

This study is also the first to evaluate 1α-hydroxylase and VDR expression in the earliest lesion associated with malignant transformation in the colon, ACF. ACFs were first defined in the colons of rodents exposed to carcinogens and identified soon thereafter in human colons (20). ACFs are associated with adenomatous polyp (42) and colorectal cancer formation in humans (20) and have become an accepted biomarker for assessing the efficacy of chemopreventive drugs in various nonhuman colorectal cancer models (43). Whereas polyps are relatively fixed as lesions, ACF seem fluid and can readily change in number over time (44). Thus, our finding that ACFs, as well as premalignant polyps, express high levels of 1α-hydroxylase and VDR, suggest that pro-vitamin D may be of value as a chemopreventive agent.

In summary, we herein show that whereas (a) 1α-hydroxylase is present in uniformly high levels in normal and neoplastic colonic epithelia, except for tumor cells metastatic to regional lymph node; (b) VDR expression is low in normal colonic epithelial cells, increases with malignant transformation and then declines with progressive tumor de-differentiation. Lastly, we show that (c) declining ratios of VDR expression in the nucleus compared with the cytoplasm in neoplastic lesions suggests that less of this protein translocates to the nucleus as tumors progress. Overall, then, these data support using pro-vitamin D analogues in colorectal cancer chemoprevention, whereas they are less likely to be useful for colorectal cancer chemotherapy.

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REVIEW

Clinical Trials Involving Vitamin D Analogs in Prostate Cancer

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ABSTRACT

Vitamin D shows significant potential as a therapy for prostate cancer. However, its use in clinical trials has been hampered by its induction of hypercalcemia at serum concentrations required to suppress cancer cell proliferation. This has spurred the development of less calcemic analogs of vitamin D. In this article, we review the clinical trials and consider the future directions of the use of vitamin D and its analogs in the treatment or chemoprevention of prostate cancer. First, we summarize the epidemiological evidence leading to the hypothesis that vitamin D has anticancer activity. We then review the clinical trials using vitamin D analogs that involve patients with prostate cancer and conclude with a brief overview of our planned study with vitamin D_5, [1α(OH)D_5], which will begin shortly. Data for this review were identified by searches of PubMed, the Cochrane Library, Biosis, and references from relevant articles, using the search terms "vitamin D," "prostate cancer," "chemoprevention" and "vitamin D analog." Abstracts from recent international meetings were also reviewed but were only included when they were the only known reference to the clinical trial or the research mentioned. Only papers published in English were included. *(Cancer J 2005;11:362–373)*

KEY WORDS

Prostate cancer, vitamin D, chemoprevention

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Prostate cancer is the most commonly diagnosed male malignancy, and the American Cancer Society has estimated that approximately 230,000 new prostate cancers will be diagnosed and about 30,000 men will die of the disease in 2004.[1] There is now good evidence that vitamin D can play a chemopreventive and/or therapeutic role in the management of prostate cancer, and in the past decade several strong reviews have addressed some of the basic biology and clinical evidence underlying the interactions of vitamin D and its analogs in prostate cancer.[2–7] Our primary aim in this paper is to review the clinical trials of vitamin D and its analogs in the treatment or chemoprevention of prostate cancer and to consider its future directions. Chemoprevention is an intervention in the carcinogenic process, possibly by a synthetic compound, which blocks, arrests, or reverses the progression of cancer.[8,9] Such chemoprevention can occur either before cancer develops (primary chemoprevention) or after a patient has had cancer and been treated for it (secondary chemoprevention) (Fig. 1). In the latter instance, the goal of chemoprevention is to slow or prevent the recurrence of cancer, with minimal toxicity to the patient. Because prostate cancer is typically a very slow-growing cancer that most often occurs in men older than 50 years of age, any therapeutic strategy that can inhibit initial tumorigenesis, delay tumor progression, or possibly induce tumor regression could reduce disease morbidity and mortality in an aging population of patients. As primary chemoprevention of cancer has yet to be well established, and given the growing life expectancy of the general population, it is likely that increasing proportions of the older male population will be living far longer with the diagnosis of prostate cancer. Thus, minimizing disease progression through secondary chemoprevention in the older patient will likely play an increasing role in the therapeutic spectrum.

It is now well established that the active metabolite of vitamin D, calcitriol, or 1,25$(OH)_2D_3$, regulates cell growth and differentiation in various in vitro can-

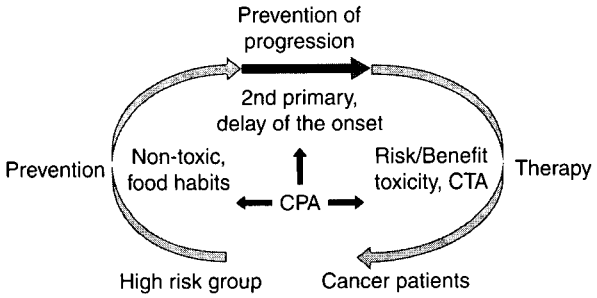

FIGURE 1 Schematic diagram for possible chemopreventive and chemotherapeutic role for vitamin D analogs. It is generally accepted that the chemopreventive agents (CPA) must be nontoxic in nature and are selective for the people at high risk for developing tumors. On the other hand, the known chemotherapeutic agents (CTA) are toxic and have major side effects. However, in this case, often the risk-to-benefit ratio is favorable towards the benefit and the side effects are generally acceptable. It is not clear when the prevention ends and therapy begins; this is represented in the figure by a gray arrow. Here one can use chemopreventive agents. In addition, if a chemopreventive agent also provides antiproliferative effects on the cancer cells it can be used as an adjuvant nontoxic chemotherapeutic agent.

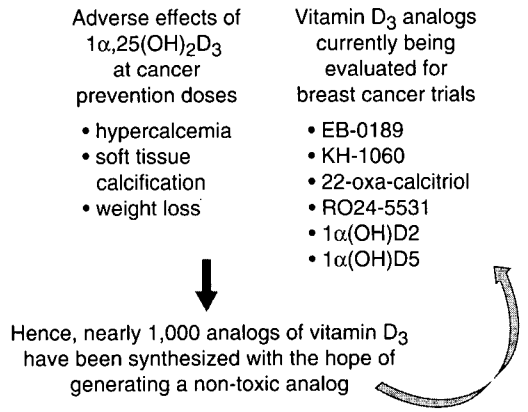

FIGURE 2 The need for analogs of vitamin D for prevention and therapy of cancer.

cer models.[10] However, the hypercalcemia it can induce at pharmacological doses continues to preclude its widespread clinical application in vivo.[11,12] Consequently, several less calcemic vitamin D analogs have been designed, synthesized, and evaluated for their chemopreventive efficacy in experimental models of carcinogenesis.[13] A number of clinical trials have also examined or are currently examining the role of vitamin D or its less calcemic analogs in treating tumors or in supplementing/potentiating chemotherapy (Fig. 2). In some of these trials, moreover, the vitamin D analogs have demonstrated an ability to curtail the growth of prostate cancer while avoiding hypercalcemia.[14] Although this review generally covers the many clinical trials involving the use of vitamin D analogs in the treatment of prostate cancer, we first summarize the epidemiological evidence that led to the hypothesis that vitamin D may have an intrinsic anticancer activity. This is followed by the in vivo and in vitro experimental evidence that one such vitamin D analog, $1\alpha(OH)D_5$, or "D_5," could prove to be useful as a chemopreventive agent for prostate cancer, based on its relative nontoxicity and its inhibitory effect in experimental carcinogenesis models. We thereafter conclude by comprehensively reviewing the clinical trials using various vitamin D analogs specifically for prostate cancer and briefly discussing our proposed clinical trial that will use vitamin D_5 at the Cancer Center of the University of California, Davis.

HISTORIC AND EPIDEMIOLOGICAL BACKGROUND

General

That adequate vitamin D intake/synthesis may prevent the development of certain diseases, such as rickets, osteoporosis, and tuberculosis, and even specific cancers, such as those of the colon, breast, and prostate, has been fairly well documented.[15–23] Indeed, it was early 20th century epidemiological evidence that first suggested an association between vitamin D and cancer protection in general, and between the risk of prostate cancer and decreased vitamin D levels specifically.

In 1936, Peller[24] initially observed that in occupations and environments in which skin cancer rates were high, the rates for other cancers were low. Subsequently, Apperly[25] also noted that populations living farther from the equator had higher overall cancer death rates compared with those living close to the equator, suggesting that increased sun exposure—and with it, an increased production of vitamin D—led to decreased cancer rates. Garland and Garland,[26] after observing that the highest death rates from colon cancer were in the northern states where there is less sunshine, hypothesized that some cancers could be associated with decreased vitamin D synthesis consequent to inadequate exposure to sunshine. They also speculated that vitamin D itself may have some anticancer properties, perhaps through the inhibition of malignant cell growth. Most recently, this observation regarding the extent of sunshine exposure and vitamin D has been extended to the incidence of as many as 13 different types of cancers.[27] Thereafter, other investigators also recognized that the proliferation, differentiation, and spread of tumor cells could be affected by $1,25(OH)_2D_3$. Over the past 20 years, extensive research involving human cell

culture has shown that vitamin D plays an important role in controlling cell growth and maturation, and that colon, prostate, and breast cells carry a protein receptor for calcitriol, the biologically active form of vitamin D.[28–34] The therapeutic potential of $1,25(OH)_2D_3$ for various types of cancer has also been noted.[29] Vitamin D inhibits the growth of normal prostatic cancer cells, many cancer cell lines, and primary cultures of prostate cancer cells.[2,35–37] In addition, in vivo animal studies have provided evidence that vitamin D and its analogs can reduce the incidence of several cancers.[38–42] Other preclinical investigations have suggested that vitamin D analogs may also inhibit tumor growth in rodents and sensitize prostate cancer cells to chemotherapeutic agents such as paclitaxel and platinum-based drugs.[40,41,43–49]

Prostate Cancer and Vitamin D

Based on the epidemiological data referenced earlier, Schwartz and Hulka[17] first proposed that vitamin D deficiency or its inadequate synthesis, a condition that can exist in the three major prostate cancer populations—older patients, African American patients, and patients living in northern latitudes—may contribute to either the initiation or the progression of the disease. Subsequent studies by Schwartz and his associates confirmed the geographic patterns of prostate cancer mortality, which show a north-to-south trend, with lower mortality rates in the South, where ultraviolet light exposure is highest. This data suggested that increased ultraviolet radiation [UVR] exposure may protect men from prostate cancer.[19] Studzinski and Moore[50] similarly speculated that sunlight exposure might afford protection against the development of prostate, colon, and breast cancer, with the recommended daily allowance of sunlight exposure varying considerably depending on degree of skin pigmentation and ethnicity. Strange and colleagues, to extend Studzinski's work in prostate cancer, compared the cumulative UVR exposure of their urology clinic patients with either sporadic prostate cancer or benign prostatic hypertrophy (BPH). Their assessment indicated that low levels of UVR exposure were significantly associated with an increased risk of developing prostate cancer.[51,52]

Clinical evidence supporting the influence of vitamin D on the risk of developing prostate cancer came from a retrospective case-control study wherein it was shown that the risk of prostate cancer was related to serum levels of vitamin D [$1,25(OH)_2D_3$] or its precursors. These sera had been collected between 1964 and 1971 from men in Northern California who later developed prostate cancer.[53] Serum samples from 90 black and 91 white men diagnosed with prostate cancer were compared with those from age-, race-, and day-of-storage-matched controls. In the men diagnosed with prostate cancer, the mean serum vitamin D level was discovered to be ~1.81 pg/mL lower than that in the matched controls ($P = 0.002$). In men > 57 years of age, moreover, low serum levels of calcitriol were found to correlate well with the risk of developing prostate cancer. In a similar evaluation involving Finnish men, Ahonen et al,[21] described an inverse association of serum $25(OH)D_3$ with prostate cancer risk. In addition, a significantly increased risk of nonlocalized prostate cancer was linked to low $25(OH)D_3$ levels in men < 52 years of age.

Traditionally, it has been held that the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ occurs primarily in the kidneys *via* the enzyme 1α-hydroxylase. Although researchers have demonstrated that exogenous $1,25(OH)_2D_3$ can delay or stop proliferation of human prostate cancer cells in culture,[43,54,55] attention has also been focused on detecting intrinsic 1α-hydroxylase activity in target organ tissues. Schwartz[56] for instance, using DU 145 and PC-3 prostate cancer cell lines, as well as primary prostate cell cultures from benign prostatic hyperlasia, has shown that prostate cells also have intrinsic 1α-hydroxylase activity and can synthesize $1,25(OH)_2D_3$ from $25(OH)D_3$. That $25(OH)D_3$ itself may inhibit cell growth in primary prostatic cell cultures,[57] coupled with results from the Finnish study[21] alluded to earlier, have fueled proposals that there may also be a potential chemopreventive role for the prohormone $25(OH)D_3$ or its analogs in prostate or other can-cers. Indeed, similar growth inhibitory effects of $25(OH)D_3$ have also been demonstrated in pancreatic adenocarcinoma cell lines[58] and, thus, potentially exist in other cancers wherein vitamin D has been postulated to play an inhibitory role. The growth effects of vitamin D appear to be mediated through the vitamin D receptor [VDR], in the prostatic cells. However, intrinsic polymorphisms in these receptors may also contribute to the effects of vitamin D seen in these studies. For instance, Luscombe et al,[59] reported an association of the *TYR A2A2* VDR genotype with reduced prostate cancer risk in men with the highest UVR exposure. In addition, the *CDX*-2 GG VDR variant is reportedly also linked to a reduced prostate cancer risk that varies with the extent of UVR exposure.[60]

While all these studies show good evidence of association between vitamin D and its prohormone with prostate cell growth regulation, there have also been other investigations in which little or no association between vitamin D and prostate cancer was identified.[61–63] For instance, Braun and associates[63] reported that no differences in prediagnostic serum $25(OH)D_3$ or $1,25(OH)_2D_3$ levels existed between

prostate cancer and control cases (34.3 vs 33.2 ng/mL or 41.0 vs 40.1 pg/mL, respectively). However, in this study, of the ~20,300 individuals from whom sera had initially been collected, only 61 developed prostate cancer 6–18 years later. Another nested case-control study using prediagnostic serum samples collected as part of the Physicians' Health Study, also reported findings wherein the median serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ were indistinguishable between prostate cancer cases and controls.[62] However, the authors acknowledge that any small to moderate beneficial effect of vitamin D or its metabolites could not be completely excluded. Nomura et al,[61] similarly analyzing serum samples exclusively from Japanese-American individuals (~3,700), also arrived at a comparable conclusion, although they conceded that the small number of study subjects may have limited the power of the study.

Thus, our knowledge of this area in prostate cancer is still incomplete despite epidemiological, basic science, and clinical advances. Although they may impede a complete current understanding of the role of vitamin D in prostate cancer, these areas of contention nonetheless represent potentially rewarding areas for continued investigation from both clinical and basic science perspectives.

VITAMIN D_5 [1α(OH)D_5]

In 1997, we synthesized a vitamin D_5 series analog, 1α-hydroxy-24-ethyl-cholecalciferol, or 1α(OH)D_5. The structures of $1,25(OH)_2D_3$ and 1α(OH)D_5 are depicted in Figure 3. This analog has been extensively evaluated in both in vitro and in vivo preclinical studies. In breast cancer cell lines, for example, any effect of vitamin D_5 on cell differentiation or apoptosis was dependent on the presence of VDR or the ability to induce VDR expression. Moreover, the absence or presence of estrogen receptors [ER] and progesterone receptors [PR] influenced the VDR-mediated effects on the cell.[64–66] In ER-positive, PR-positive, and VDR-positive cell lines (BT474, MCF7, or T47D), the vitamin D analog induced cell differentiation as well as apoptosis, whereas in ER-negative, PR-negative, and VDR-positive cells (BCA-4), 1α(OH)D_5 induced only cell differentiation.[64,65] Thus, the effects of the new D_5 analog, insofar as the VDR requirement is concerned, appeared to be comparable to those described for $1,25(OH)_2D_3$. We have also evaluated the D_5 analog's chemopreventive activity in carcinogen-treated murine mammary glands in organ culture experiments,[67,68] wherein the compound significantly inhibited the development of carcinogen-induced ductal or alveolar mammary lesions. In addition, vitamin D_5 treatment also resulted in epithelial cell death in

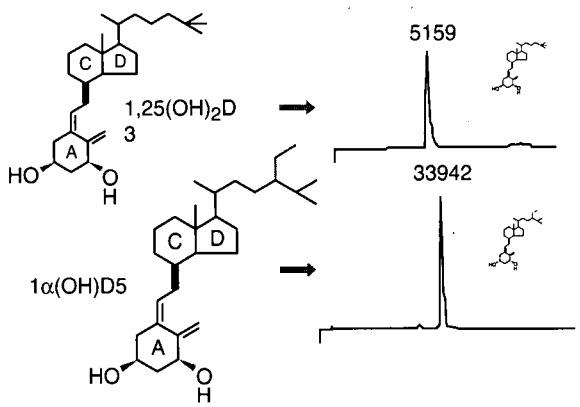

FIGURE 3 Structures of $1,25(OH)_2D_3$ (top) and 1α(OH)D_5 (bottom).

human breast tumor explants but not in epithelial cells from normal breast tissue explants.[67] In in vivo analyses as well as in in vitro explant cultures involving Sprague-Dawley rats, treatment with vitamin D_5 reduced the incidence of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumors and tumor multiplicity.[69] Although both the tumor initiation and tumor promotion stages were impeded by vitamin D_5 treatment, it was more selective for the promotion stage of carcinogenesis.[70] Thus, vitamin D_5 treatment was associated with a reduced incidence of mammary carcinogenesis in vivo and appeared to have significant potential as a chemopreventive agent.

In initial studies of the calcemic toxicity of 1α(OH)D_5 using vitamin D deficient Sprague-Dawley rats, it was demonstrated that the 1α(OH)D_5 did not induce significant hypercalcemia at doses up to 50 μg/kg.[69] We also recently completed a thorough preclinical toxicity study to determine dose tolerance in beagle dogs and CD rats. The rats received 28 days of gavage feeding treatment, with increasing doses of 1α(OH)D_5 in a range of 2.5–10 μg/kg body weight for CD-1 rats and 5–50 μg/kg body weight for beagle dogs. A complete battery of in-life, clinicopathologic, and histopathologic evaluations were performed on the treated animals. No toxicity or enhanced serum calcium levels were observed in the rats at any of the test doses. In beagle dogs, doses up to 5 μg/kg body weight also resulted in no measurable toxicity. However, administration of greater than 10 μg/kg body weight resulted in weight loss, increased serum calcium, and gross toxicity (unpublished data).

Taken together, our in vitro and in vivo investigations suggest that this new vitamin D analog may have strong value for chemoprevention of breast and other hormone-modulated cancers. It appeared to be nontoxic at a wide range of doses, appeared to mediate its effects through VDR, induced cell cycle arrest and apoptosis in transformed cells, and inhibited

ductal and alveolar breast carcinogenesis in vivo and in vitro. These findings warranted deeper investigations of the effects of vitamin D_5 in other cancer cell types, such as prostate cells and/or prostate cancer tissue explants, as well as in phase I/II clinical trials involving patients with breast or prostate cancer. Such preclinical studies involving $1\alpha(OH)D_5$ in prostate cancer are now in progress and a phase I chemoprevention trial for breast cancer and prostate cancer has been proposed. Before discussing the salient aspects of the proposed phase I/II clinical trial for prostate cancer, however, we first review the clinical trials performed to date with analogs of vitamin D.

CLINICAL TRIALS OF PROSTATE CANCER WITH VITAMIN D ANALOGS

Vitamin D shows great potential as a therapeutic intervention for prostate cancer. However, its usefulness in clinical practice has been hindered by its induction of hypercalcemia at a concentration required to suppress cancer cell proliferation.[13,71] Over 2,000 analogs of vitamin D have been synthesized and evaluated during the past 10 years, with the hope of generating any that would exhibit reduced calcemic activity without compromise of antiproliferative activity, in comparison with vitamin D.[13,32,72] Several relatively less toxic but somewhat efficacious analogs have already been evaluated in preclinical toxicity and phase I or II clinical trials for dose tolerance in patients with advanced cancer. The best studied of these analogs or precursors include 1,25-dihydroxy D_3, 22-oxa-calcitriol, EB1089 [seocalcitol, an analog of $1,25(OH)_2D_3$], calcipotriol, KH1060, and R024-5531. These agents have been considered acceptable for clinical trial use based on their low preclinical toxicity in animals under stringent experimental conditions. One small study has also evaluated the usefulness of cholecalciferol, a vitamin D precursor, in prostate cancer.[73] The nontoxic, well-tolerated doses for calcitriol and other analogs of vitamin D that have been used are elaborated in Table 1. In addition to analog variety, clinical trials have also examined a variety of drug administration schedules with the vitamin D analogs, in order to avoid or minimize the toxicity associated with pharmacological doses of the vitamin D analogs. One means was to examine the effects of administering calcitriol only, given daily, for patients with advanced prostate cancer. Another variation with calcitriol was to adhere to an intermittent dosing schedule and/or to use alternative routes of administration. Investigators have also tried administering calcitriol in lower than usual doses but in combination with other drugs, hoping to minimize the toxicity of calcitriol while anticipating synergism with or potentiation of calcitriol's therapeutic effects by the coadministered drugs.

Besides calcitriol, a vitamin D_2 analog has also been studied as monotherapy, administered daily in patients with advanced prostate cancer. Finally, our planned study with D_5 examines the effects of the new vitamin D analog and evaluates its long-term effects and toxicities when administered daily to prostate cancer patients who have been treated for their cancer with radiation therapy (RT). Overall, there have been at least 13 vitamin D-related clinical trials to date (see Table 1), exploring different study drugs or variations in administering those drugs in an effort to determine the optimal combination/dose for chemoprevention of prostate cancer.

In 1995, Osborn et al[74] conducted the first clinical trial using vitamin D in the treatment of prostate cancer. They carried out a small phase II trial of calcitriol (Rocaltrol) $[1,25(OH)_2D_3]$ in 13 evaluable patients with hormone-independent prostate cancer. The $1,25(OH)_2D_3$ was administered daily as an oral dose of 0.5 μg, and this was escalated gradually to a dose of 1.5 μg daily if no toxicity was encountered. Eleven patients reached the maximum dose of 1.5 μg daily. Only two patients had decreases in their serum PSA levels after taking $1,25(OH)_2D_3$, and grade 1 hypercalcemia was the main dose-limiting toxicity in the 11 patients, as assessed by the National Cancer Institute [NCI] Common Toxicity Criteria. However, no anti-tumor effect of calcitriol was observed. The authors concluded that $1,25(OH)_2D_3$, as administered in their study, was ineffective in treating advanced prostate cancer, although in two patients a decrease in serum PSA levels was noted. However, it is conceivable that the patients with less advanced disease may benefit from treatment with vitamin D or one of its analogs. Subsequently, in another small pilot clinical trial involving patients with advanced prostate cancer, calcitriol alone was administered daily to determine if it would slow the rate of PSA doubling.[12,75] Following radical prostatectomy or radiation therapy, these patients experienced biochemical failure. However, they were ineligible for androgen ablation therapy. Seven patients completed 6–15 months of dose escalation calcitriol therapy. The treatment was initiated with 0.5 μg daily and was increased weekly, as tolerated, to a maximum dose of 2.5 μg daily. Calcitriol administration reduced the rate of PSA doubling in 6 out of the 7 patients. It did not appear to be tumoricidal, but it did slow tumor cell growth, showing for the first time that vitamin D or its effective analogs could be clinically helpful in treating patients with prostate cancer postradiotherapy or postradical prostatectomy. The study also suggested that a 1.5-μg

dose of $1,25(OH)_2D_3$, generally considered the most toxic of the vitamin D analogs, was tolerated by patients, with doses beyond 1.5–2.5 μg limited by the resulting hypercalciuria, although no patient experienced hypercalcemia at the doses administered in this study. When the therapy was discontinued, the patients' PSA doubling times reverted to the pretreatment indices. Because the calcemic effects of daily calcitriol limited any dose escalation in this trial,[12,75] as well as in the trial by Osborn et al,[74] other investigators decided to examine the roles of intermittent administration, other routes of administration, and other vitamin D analogs.

Administration of calcitriol subcutaneously, every other day (QOD), was tested for the first time in a phase I study carried out in patients with advanced solid tumors, to evaluate the pharmacokinetics and determine a maximum tolerated calcitriol dose via this route.[11] Thirty-six patients, most of whom had prostatic adenocarcinoma, were treated with subcutaneous calcitriol at doses ranging from 2–10 μg QOD. The maximum tolerated dose was 8 μg, and none of the six patients tolerating this dose developed hypercalcemia. However, dose-limiting hypercalcemia occurred in all three patients who received the highest dose (10 μg). The subcutaneous doses administered in this study were significantly higher than the oral 2.5-μg dose achieved in the Gross et al study[12,75] or the 1.5-μg dose in the Osborn et al study.[74] At all the dose levels, moreover, hypercalciuria occurred; hypercalcemia, however, occurred only at the 10-μg dose. Some patients with stable disease were able to tolerate this therapy at the lower doses for up to 4 months. Most patients, however, developed progressive disease despite the therapy and discontinued it much earlier. Also, six of the patients who had hormone-refractory prostate cancer and received doses of 6 μg or less did not show any clinical response to the treatment. No significant anti-tumor responses were shown in this trial, but the therapy was generally well tolerated. The results of this study suggested that vitamin D and its analogs could be used at a relatively high dose and for a relatively long time period when administered subcutaneously QOD, and pointed to a need for further testing with calcitriol and vitamin D analogs for treating prostate cancer.

Beer and colleagues[7] presented the first clinical evidence that vitamin D might improve the effectiveness of chemotherapy in prostate cancer. They tested intermittent doses of calcitriol, alone or with other treatments, such as docetaxel, in patients with advanced prostate cancer. Intermittent dosing was selected to test the hypotheses that exposing tumor cells briefly to calcitriol would result in prolonged antiproliferative effects without the development of hypercalcemia, and thus allow large escalations of calcitriol doses. From in vitro studies involving calcitriol, it had been shown that peak serum calcitriol concentrations of at least 1 nM had to be attained in order to inhibit prostate cancer proliferation by more than 50%.[40,41,75] Thus, meeting or exceeding this level in serum has been a goal in their series of clinical trials. Nonetheless, it should be noted that the clinically equivalent peak serum concentrations of other vitamin D analogs could be different.

In the first of a series of trials of intermittent calcitriol, Beer et al[76] treated 15 patients with advanced prostate, breast, lung, or other cancers, once weekly

TABLE 1 Studies with Prostate Cancer Patients and Vitamin D Analog Treatments

Author	Year	No. of Patients	Therapy	Dose/Frequency	Duration
Beer et al (ASCENT)[84]	2004	250	Calcitriol + docetaxel	0.5 μg/kg, qw	8+ weeks
Beer et al[77]	2003	22	Calcitriol	0.5 μg/kg, qw	2–25+ months
Beer et al[78]	2003	37	Calcitriol + docetaxel	0.5 μg/kg, qw	8–93+ weeks
Liu et al[87]	2003	20	$1\alpha(OH)D_2$	12.5 μg, qd	3–108 weeks
Liu et al[86]	2002	25	$1\alpha(OH)D_2$	5–15 μg, qd	8+ weeks
Muindi et al[81]	2002	36	Calcitriol + paclitaxel	4–38 μg, qdx3	Unknown
Beer et al[76]	2001	15 (5 with PCa)	Calcitriol	0.06–2.8 μg/kg, qw	16+ weeks
Johnson et al[79]	2000	43	Calcitriol + carboplatin	4–13 μg, qDx3 q28 days	3+ months
Johnson et al[79,80]	2000	Unknown	Calcitriol + paclitaxel	4–38 μg, qDx3, weekly	Unknown
Trump et al[82]	2000	35	Calcitriol + dexamethasone	8–12 μg, qDx3	3+ months
Smith et al[11]	1999	36 (6 with PCa)	Calcitriol	2–10 μg, qOD	Up to 4 months
Gross et al[12] and Krishnan et al[92]	1998 / 2003	7	Calcitriol	0.5–2.5 μg, qD	6–15 months
Osborn et al[74]	1995	13	Calcitriol	0.5–1.5 μg/qD	4+ weeks

Abbreviations: PCa = prostate cancer.

for 4 weeks, with an oral pulse-dose of calcitriol starting at 0.06 μg/kg, followed by 4 weeks of observation with no further treatment. Five of these 15 patients had adenocarcinoma of the prostate. Patients could continue to receive treatment, with dose escalations, for additional 8-week cycles, provided they had no tumor progression or grade 3 or higher toxicity (NCI Common Toxicity Criteria). Doses as high as 2.8 μg/kg were achieved in this study without any dose-limiting toxicity or hypercalcemia, and weekly oral-dose calcitriol was found to be quite safe for dose escalation and achievement of peak serum calcitriol levels without creating significant toxicity. A dose of 0.5 μg/kg was selected for follow-up phase II trials on the basis of this phase I trial.

Beer et al[77] also studied the long-term toxicity of weekly dosing of oral calcitriol in patients with recurrent prostate cancer, most of whom had not received any hormone therapy. Twenty-two patients in this phase II trial received calcitriol in pulse doses of 0.5 μg/kg weekly, for periods ranging from 2 to >25 months, while maintaining a reduced calcium diet. Patients experienced no hypercalcemia, clinically significant renal calculi, or grade 3 or higher toxicity. The investigators defined the efficacy of this treatment as, "≥50% reduction in serum PSA," but no patients achieved that endpoint. However, there were indications that the treatment regimen had some therapeutic effect, as three patients showed some reduction in serum PSA (47%, 28%, and 10%) and PSA doubling time was reportedly increased in three other patients.

Subsequently, an investigation of the efficacy and long-term toxicity of high-dose weekly oral calcitriol plus docetaxel was carried out by Beer and colleagues.[78] In this phase II trial, 37 patients with metastatic, androgen-independent prostate cancer were treated with weekly oral calcitriol in doses of 0.5 μg/kg on day 1, followed by docetaxel (36 mg/m^2) intravenous administration on day 2. This weekly regimen was repeated for 6 weeks of an 8-week cycle (i.e., a 2-week rest period). Patients also adhered to a reduced calcium diet during the treatment weeks. Treatment continued until prostate cancer progression or excessive toxicity or PSA was <4 ng/mL. Thirty patients (81%) experienced a reduction in PSA (defined as ≥50% reduction of serum PSA). For 11 patients, prostate cancer was held in check for a median period of about 1 year (range, 29–93 weeks). The combination of weekly oral high-dose calcitriol and weekly docetaxel was well tolerated and effective in this single, nonrandomized trial, with no grade 3 hypercalcemia or significant toxicity reported. A randomized prostate cancer trial examining the effect of calcitriol and docetaxel against a placebo is now in progress.[75] Besides these investigations with calcitriol alone, either orally or subcutaneously, given daily or on intermittent dosing schedules, calcitriol has also been combined with other therapies in several studies. For example, Johnson and colleagues[79,80] conducted two phase I trials of calcitriol in combination with one of two cytotoxics, either carboplatin or paclitaxel, in men with androgen-independent prostate cancer. In the first trial, carboplatin was administered every 28 days, with subcutaneous calcitriol administered daily for the first 3 days of each week. The calcitriol starting dose was 4 μg per day. No dose-limiting toxicity was seen and doses of calcitriol as high as 13 μg were achieved. In the second trial,[79] paclitaxel was given with orally administered calcitriol. The starting dose of calcitriol was 4 μg, and some patients eventually received a dose as high as 38 μg. Again, no dose-limiting toxicity was seen. The studies continued with patients receiving calcitriol doses of 14 μg in a liquid formulation. The results of these two trials suggested that calcitriol could be well tolerated when combined with other chemotherapeutic agents used to treat androgen-independent prostate cancer.

Other clinical trials too have found that high-dose calcitriol is feasible on an intermittent dosing schedule, giving large amounts to cancer patients just 3 days a week.[81] Phase II trials were also initiated to test administration of calcitriol plus dexamethasone to patients with androgen-independent prostate cancer, one of several studies investigating combining calcitriol with cytotoxic agents.[82] Dexamethasone had enhanced calcitriol anti-tumor efficacy in preclinical studies, leading to its selection for a clinical trial.[83] Calcitriol dosing was begun at 8 μg, 3 times a week (Monday, Tuesday, and Wednesday), for 4 weeks; then, assuming no toxicity occurred, the dose was raised to 10 μg for 1 month. Thereafter, if no toxicity occurred, 12 μg of calcitriol was administered for the rest of the study. Patients also received 4 mg of dexamethasone orally for 4 days each week (Sunday, Monday, Tuesday, and Wednesday). Of the 43 patients treated, 35 had received 12 μg, 3 times a week, for more than 1 month. None of the patients needed dose reductions secondary to hypercalcemia. Some significant anti-tumor responses were observed in the patients in this study; 80% of patients had a decrease in the rate of increase in PSA, and 34% showed stable or decreased PSA (>50% reduction). These data suggested that treating patients with androgen-independent prostate cancer with high-dose calcitriol and dexamethasone could be safe and clinically effective, although the potential contribution of the high dexamethasone doses *per se* to these results must also be considered.

Other investigators too have examined the effectiveness of combining calcitriol with other chemotherapy agents. For instance, Muindi and colleagues[81] reported on experience in determining the maximum tolerated dose of calcitriol, in combination with paclitaxel, in patients with advanced colorectal, lung, prostate, and other cancers. Thirty-six patients were treated. The starting dose of calcitriol was 4 μg, for 3 consecutive days each week, and the maximum achieved dose was 38 μg. No dose-limiting toxicity was recorded in the study, and the investigators concluded that high doses of calcitriol could be safely administered in combination with paclitaxel.

Another clinical trial using vitamin D in combination with a cytotoxic drug to treat prostate cancer is the ASCENT (AIPC Study of Calcitriol Enhancing Taxotere) trial. This trial, also led by Beer and colleagues[75,84] and following up on their earlier clinical trials, has enrolled ~250 men at 58 centers across the United States and Canada. This phase II and III trial is a placebo-controlled, double-blinded, randomized comparison of DN-101 in combination with docetaxel, to docetaxel alone, in patients with advanced prostate cancer and in whom hormone therapy has failed. The study drug will be administered weekly. DN-101 is a highly concentrated oral formulation of calcitriol designed specifically for cancer therapy that may potentially allow for greater dose escalation compared with standard oral calcitriol. To date, dose levels ranging from 15–165 μg have been tested, and 45 μg has been selected as the maximum tolerated dose for further studies. Eighteen patients have taken that dose for from 3 to > 40 weeks without any dose-limiting toxicity. If the trial results in positive outcomes, it will promote a new, well-tolerated treatment regimen for advanced prostate cancer. Enrollment in the trial is complete, and results are anticipated in late 2004 or early 2005.

Although calcitriol has been the main vitamin D analog studied in clinical trials, other investigators have used a different analog, vitamin D_2, with some patients. These trials were suggested by results from pilot studies, including those of Wigington et al,[85] wherein the growth inhibitory effects of $1,24(OH)_2D_2$ were demonstrated on androgen-responsive lymph node-prostate cancer (LNCaP) cells. The first clinical trial involving $1\alpha(OH)D_2$, or D_2, was conducted by Liu et al.[86] Developed by BCI (Madison, WI), D_2 is an inactive precursor of two naturally occurring hormones, $1\alpha,25(OH)_2D_2$ and $1\alpha,24(OH)_2D_2$. The analog was studied in preclinical trials and pilot clinical trials for treatment of conditions other than prostate cancer before it was used in a prostate cancer clinical trial. Liu et al have completed a phase I, dose-escalation trial of $1\alpha(OH)D_2$, a vitamin D analog, in patients with hormone-refractory prostate cancer.[86] The $1\alpha(OH)D_2$ was administered in doses of 5–15 μg/day to determine the maximum tolerated dose and detect any anti-tumor activity. Twenty-five patients participated in this clinical trial, with 16 patients completing ≥ 8 weeks of therapy; 11 of these 16 patients received doses of 10 μg/day or higher. The main toxicities, as anticipated, were hypercalcemia and renal insufficiency. Overall, however, $1\alpha(OH)D_2$ was well tolerated. Seven patients had an objective response to the treatment with $1\alpha(OH)D_2$, with two achieving ≥ 50% reductions in PSA and five demonstrating stable disease for more than 6 months.

Liu and colleagues[87] then followed up their phase I trial with a phase II trial involving 26 enrolled patients (20 evaluable) with advanced hormone-refractory prostate cancer, to evaluate the efficacy of the vitamin D analog $1\alpha(OH)D_2$. Patients received 12.5 μg/day of $1\alpha(OH)D_2$, which was the dose derived from the earlier phase I study. The study drug was given in five capsules of 2.5 μg each. Mild, expected toxicity occurred, including clinically insignificant hypercalcemia in some patients. However, the primary endpoint of this study was progression-free survival for 6 months and not PSA response. Overall, stable disease was maintained for an average of 19.2 weeks, with six patients achieving disease stabilization for over 6 months and one patient achieving disease stabilization for over 2 years (range, 3–108 weeks). These results, too, suggested that further trials with vitamin D analogs should be actively pursued in prostate cancer. There have been only a few chemoprevention clinical trials of vitamin D analogs in prostate cancer, and they have been typically short-term studies involving only small numbers of patients and frequently limited in dose escalation potential by vitamin D's intrinsic hypercalcemic toxicity (see Table 1). Considerable progress has been achieved through these clinical trials, however, insofar as limiting the hypercalcemic toxicity of vitamin D and its analogs. Nonetheless, dose escalation of the vitamin D analogs is still limited, although coadministered cytotoxic drugs such as docetaxel or dexamethasone, among others, may have augmented their therapeutic efficacy.

Moreover, these chemoprevention trials involving prostate cancer have generally focused on patients with advanced disease, as these patients, many with androgen-independent disease, have few other treatment options. Chemopreventive agents in this patient population are primarily intended for prolongation of survival beyond what would be possible with chemotherapy or other treatments. Thus, there is still significant room for the development and evaluation of current and new vitamin D analogs, especially if

they are to be used in adequately powered, randomized, placebo-controlled clinical trials, to evaluate efficacy in the "primary" chemoprevention of prostate cancer, as well as in "secondary" chemoprevention in patients following definitive treatment of the earlier stages of prostate cancer. Because subjects drawn from these populations are likely to have undergone definitive therapy and/or are not likely to have disease, the argument for merely using low doses of currently available vitamin D analogs because their effects can be supplemented with cytotoxic drugs for chemoprevention loses validity. Thus, the hypercalcemic effects of any vitamin D analog used in these subjects for chemoprevention must simply be minimal at pharmacological doses, and the search for such analogs continues.

PLANNED PROSTATE CANCER CLINICAL TRIAL WITH $1\alpha(OH)D_5$

It has been convincingly shown that the only major toxic effect of treatment for prostate cancer with vitamin D is hypercalcemia but based on our studies in mice, rats, and beagles, the vitamin D_5 analog $1\alpha(OH)D_5$ is comparatively nontoxic. The results of the studies involving other vitamin D analogs reviewed above suggest that for $1\alpha(OH)D_5$, which is relatively well tolerated at much higher in vivo doses in experimental models, 10 μg per day should not pose any toxicity problem in patients with prostate cancer. Secondly, as it has been shown that $1,25(OH)_2D_3$ reduces the rate of elevation of PSA in prostate cancer patients, it is anticipated that $1\alpha(OH)D_5$ will be as effective in this regard for postradiotherapy patients, with no drug-related side effects. Based on these data and our review of the Prostate Cancer Prevention Trial (PCPT)[88] and the Selenium and Vitamin E Cancer Prevention Trial (SELECT),[89] we have designed a combined phaseI/II clinical trial to evaluate the efficacy of $1\alpha(OH)D_5$ in patients previously treated for prostate cancer with radiation therapy. Because the design of this study has been recently published,[90] only the salient points are briefly reviewed here.

A critical area for future research is identification and a better understanding of vitamin D-modulated biomarkers in cancer. Selecting intermediate endpoint markers for the diagnosis, progression, or response to treatment has been a major challenge in all forms of cancer. In this respect, prostate cancer diagnosis has been considerably simplified by the identification of prostatic intraepithelial neoplasia [PIN] and serum prostate-specific antigen [PSA]. Important aspects to be considered in chemoprevention studies are serum and tissue intermediate biomarkers. After reviewing past studies and the PCPT and SELECT, we designed a clinical trial with $1\alpha(OH)D_5$. This is a phase I/II safety/chemoprevention study to determine if $1\alpha(OH)D_5$ can safely and efficaciously delay prostate cancer recurrence when administered after RT. Forty randomized patients will receive either $1\alpha(OH)D_5$ or a placebo, beginning 12–60 months after completion of definitive RT. In contrast to earlier studies with other vitamin D analogs, our study includes a placebo-controlled arm for comparison, as well as a 1-month run-in period to optimize patient compliance. Patients will receive baseline clinical staging, a pretreatment biopsy, and serum PSA measurements.

Subjects will be monitored using serum chemistries and albumin weekly in the first month. Individuals with stable calcium levels will then have weekly phone calls and monthly clinical assessments. Serum chemistries, albumin, parathyroid hormone (PTH), and urine electrolytes will be obtained monthly. Parathyroid hormone levels will be monitored biannually. Individuals with stable calcium levels at 4 months will transition to a 4-month monitoring cycle, with chemistries, albumin, PTH, and urine electrolytes drawn immediately prior to a visit. At the end of the study, subjects will receive final laboratory and clinical evaluations and undergo a prostate biopsy. Patients will continue to receive 2 years of posttreatment follow-up.

SUMMARY

While vitamin D analogs continue to be investigated for a role in the reduction of the incidence of prostate cancer progression following therapy, Peehl and Feldman[91] have proposed that this possibility might be confounded if prostate cancer cells were to develop a resistance to the tumor suppressor effects of vitamin D. The inhibitory effects of vitamin D could also be subverted by other mechanisms that prostate cancer cells have developed. For instance, over time, prostate cancer cells could lose receptors or signaling molecules that mediate the actions of vitamin D, or experience changes in metabolic enzymes that degrade vitamin D and its metabolites, making them less sensitive to the effects of vitamin D. This may be an important consideration for investigators in the future as the development of vitamin D analogs as chemopreventive agents matures. Although epidemiological studies and early clinical trials have suggested that vitamin D may have chemopreventive qualities for prostate cancer, randomized phase III clinical trials will ultimately be necessary to defini-

tively ascribe a chemopreventive effect to it or to any of its analogs. The issues then for future clinical studies include determining the optimal dose, the preferred analog, the route and schedule of administration, the patient population, and the specific aim of the trial (i.e., primary or secondary chemoprevention). The optimal vitamin D analog will be the one that is the most effective in preventing or treating cancer while also being the least calcemic, especially in regard to primary chemoprevention or secondary chemoprevention in patients with early prostate cancer. Patients at the highest risk of recurrence should be targeted for these clinical trials of chemoprevention agents. At the same time, basic studies in the laboratory need to continue in order to further elucidate the mechanisms by which vitamin D and its analogs act at the cellular and molecular level in prostate cancer.[74,93] and to identify additional targets downstream of the VDR for chemoprevention.

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Chemopreventive efficacy of 25-hydroxyvitamin D3 in colon cancer

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Abstract

Recently, it has been reported that 25-hydroxyvitamin D3-1α-hydroxylase [1α(OH)ase, CYP27B1], required to convert non-toxic 25-hyxdroxyvitamin D3 [25(OH)D_3] to its active metabolite [1α,25(OH)$_2D_3$], is present in the epithelial cells of the human colon. In the present study, the potential chemoprotective role of 25(OH)D_3 was evaluated for colon cancer using the HT-29, human colon cancer cell line. Colon cancer cells were treated with 25(OH)D_3 (500 nM or 1 μM), 1α,25(OH)$_2D_3$ (500 nM), cholecalciferol (D3, 1 μM) or vehicle and cell number determined at days 2 and 5 post-treatment. Results showed that both 25(OH)D_3 and 1α,25(OH)$_2D_3$ induced dose- and time-dependent anti-proliferative effects on the HT-29 cells, with maximum inhibition noted at day 5. Western blot analyses revealed an up-regulation of VDR and 1α(OH)ase expression following 24 h of treatment with 25(OH)D_3, and 1α,25(OH)$_2D_3$. These results are consistent with the expression of VDR and 1α(OH)ase in samples of normal colonic tissue, aberrant crypt foci (ACFs) and colon adenocarcinomas. The VDR expression was sequentially increased from normal to pre-cancerous lesions to well-differentiated tumors and then decreased in poorly differentiated tumors. Expression of 1α(OH)ase was equally expressed in normal, pre-cancerous lesions and malignant human colon tissues. The increased expression of 1α(OH)ase in colon cancer cells treated with the pro-hormone and its anti-proliferative effects, suggest that 25(OH)D_3 may offer possible therapeutic and chemopreventive option in colon cancer.

Keywords: Colon chemoprevention; 25-Hydroxyvitamin D3; VDR; CYP27B1

1. Introduction

Epidemiological studies have been instrumental in demonstrating an inverse relationship between vitamin D intake, sunlight exposure and risk for colon cancer [1]. Recently, a meta-analysis conducted by Gorham et al. [2] examined the dose response relationship between vitamin D intake or serum 25(OH)D_3 and risk for colon cancer. Dose-gradient curves generated from 18 of the 44 existing observational studies demonstrated that individuals with ≥ 1000 IU/day oral vitamin D or ≥ 33 ng/ml serum 25(OH)D_3 had 50% lower risk for developing colon cancer compared to reference values [2].

Inspite of the extensive evidence demonstrating the anti-cancer activity of 1,25(OH)$_2D_3$ in colon cancer, its clinical use is limited by its susceptibility to cause hypercalcemia. Hence, in order to retain the efficacy but reduce its toxicity numerous analogs have been synthesized and evaluated for the prevention and treatment of colon cancer. Until recently, little attention was given to 25(OH)D_3 as a potential chemopreventive agent since it was commonly believed that the enzyme 1α(OH)ase was selectively present in the kidney. The existence of 1α(OH)ase in colon epithelial cells has led to interest in the potential use of this non-toxic pro-hormone, 25(OH)D_3, as an effective chemoprotective agent against colon cancer [3–5]. The possibility of using the 25(OH)D_3 as a chemopreventive and/or chemotherapeutic agent has been further strengthened by *in vitro* studies which have demonstrated that cells containing 1α(OH)ase are able to convert 25(OH)D_3 into 1,25(OH)$_2D_3$ [6,7]. For example, Bareis et al. [7] demonstrated that the Caco-2 cells, which is a moderately differentiated colon cancer cell line, are able to produce 1,25(OH)$_2D_3$ from the pro-hormone. Here we report that normal, aberrant crypt foci (ACF) and malignant human cancer samples express VDR and 1α(OH)ase and that 25(OH)D_3 is efficacious as an anti-proliferative agent in human colon cancer cells.

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2. Materials and methods

2.1. Tumor specimens and histological grading

Colon cancers were randomly selected from the University of Illinois at Chicago Gastrointestinal Tumor Bank. The University of Illinois at Chicago and Veterans Administration Institutional Review Boards approved use of these tissues. Differentiation was assessed as previously described [8].

2.2. Human colon cancer cell lines

The HT-29, Caco-2 and SW480 cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 media (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum, 2 mM L-glutamine and 1% antibiotic–antimycotic solution and kept in a 37 °C humidified atmosphere of 5% CO_2.

2.3. Analysis of cell proliferation

For determination of proliferation, HT-29 cells were seeded at a density of 2×10^4 per well in a 12-well cell culture plate and allowed to adhere overnight. After incubation with or without 25(OH)D_3 for the appropriate times, cells were detached with trypsin and cell number was determined by the Coulter counter.

2.4. FACS analysis

Colon cancer cells were seeded at a density of 5.0×10^5 in 25 cm^2 flasks and allowed to adhere for 24 h. Following treatment with or without 1.0 μM 25(OH)D_3 for 48 h, they were harvested with trypsin and washed with PBS. The samples were then stained with propidium iodide using the detergent-trypsin method described by Vindelov et al. [9].

2.5. Measurement of apoptosis

Cells undergoing apoptosis were evaluated using the In Situ Cell Death Detection Kit (Roche, Indianapolis, IN). A quantitative assessment was made by determining the percentage of apoptotic cells.

2.6. Western blot analysis

Treated and untreated cells were lysed in freshly prepared extraction buffer. Protein concentration was determined using a modified Lowry method (Bio Rad, Hercules, CA). Samples were then separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked and then incubated with appropriate primary and secondary antibodies. Anti-VDR antibody was from Neomarkers (Freemont, CA), sheep anti-murine 25-hydroxyvitamin D3-1α-hydroxylase antibody was from the Binding Site (San Diego, CA). The chemiluminescence reaction was performed using the ECL system. Bands of interest were compared to that of actin and relative intensity ratios were calculated.

2.7. Immunofluorescence studies

SW480 cells were seeded on cover slips and allowed to adhere overnight. After incubation with or without 25(OH)D_3 (1 μM) for 24 h, the cells were fixed in buffered formalin, washed with PBST (PBS containing 0.1% Tween 20), permeabilized in 0.2% Triton X-100/PBS, blocked with 1% BSA, and then incubated with anti-VDR rat monoclonal antibody (1:200) for 1 h at RT. Cells were washed and incubated with TRIC-labeled anti-rat secondary antibody for 1 h. After staining nuclei with DAPI, cells were visualized using the Olympus BX51 microscope. Tissues were sectioned (4 μm thick) and processed for immunohistochemistry as previously described [10].

3. Results

3.1. Expression of 1α(OH)ase and VDR in human colon tissue and cancer cells

The expression patterns of VDR and 1α(OH)ase were evaluated in human colon tissues. As shown in Fig. 1A–E, 1α(OH)ase showed consistently strong expression in normal, premalignant (ACF) and malignant colonic epithelial cells, irrespective of tumor cell differentiation. In contrast, minimal VDR expression was observed in normal colonic epithelial cells, with the expression localized predominantly in the nucleus (Fig. 1F). Samples revealed that the amount of VDR is significantly higher in ACFs than to that of adjacent normal tissue. Moreover, with malignant transformation, total cellular VDR expression increased markedly and was especially high in well-differentiated tumors (Fig. 1H and I) but then decreased with tumor cell de-differentiation (Fig. 1J). Furthermore, these data suggest that 1α(OH)ase (Fig. 1B) and VDR (Fig. 1G) expression levels in colorectal cancer pre-cancerous lesions such as ACFs and polyps may permit the pro-hormone 25(OH)D_3 for use in colorectal cancer chemoprevention.

The expression profile of VDR and 1α(OH)ase in human colon tissue prompted us to investigate whether 25(OH)D_3 treatment regulated the expression levels of VDR and 1α(OH)ase in the HT-29 cells. For this, HT-29 cells were treated with vehicle (ETOH), 25(OH)D_3 (1 μM), 1α,25(OH)$_2D_3$ (0.5 μM; positive control) or cholecalciferol (D3, 1 μM; negative control). Following 24 h of treatment, protein lysate was collected and separated for Western blot analyses. As shown in Fig. 2A, both 25(OH)D_3 and 1,25(OH)$_2D_3$ up-regulated the levels of expression of 1α(OH)ase by 22 and 30%, respectively. In a similar manner, VDR expression levels (Fig. 2B) were significantly increased following treatment with 25(OH)D_3 and 1α,25(OH)$_2D_3$ by 40 and 48%, respectively. Treatment with the cholecalicerfol

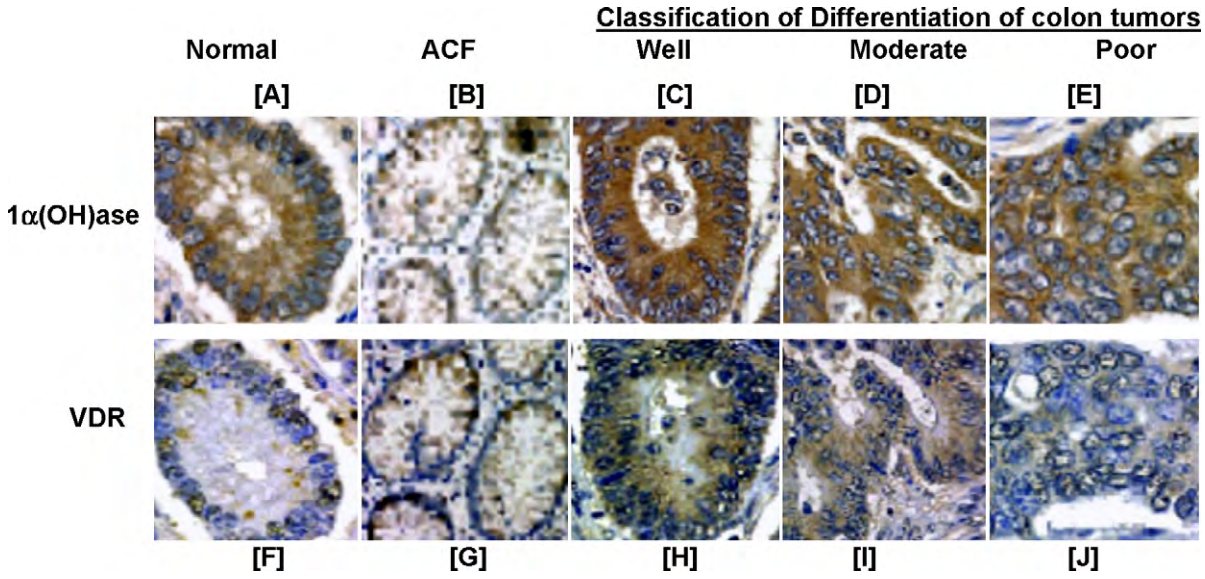

Fig. 1. Expression of 1α-hydroxylase and VDR in human colon tissue. Similar non-nuclear levels of 1α-hydroxylase expression are noted in normal colonic epithelial cells (A), aberrant crypt foci (B), as well as, in well-differentiated tumor cells (C), moderately differentiated tumor cells (D) and poorly differentiated tumor cells (E). VDR expression levels were low in normal colonic epithelial cells (F), increased in aberrant crypt foci (G) with further increase noted in well-differentiated tumor cells (H). In contrast, moderately differentiated tumor cells (I) appear to have less VDR than the well differentiated. VDR expression was found to be almost completely lost in poorly differentiated tumor cells (J). Magnification ×1000.

(negative control) had no effect on the expression levels of 1α(OH)ase (Fig. 2A) and showed only a marginal increase in the levels of VDR (Fig. 2B).

In addition to HT-29 cells, we evaluated the effects of 25(OH)D_3 on SW480 colon cancer cells. The SW480 cell line was selected for this study since it is one of the best characterized human colon cancer cell lines. Moreover, it is widely used as a model system for vitamin D compounds, and is shown to have both VDR negative [VDR(−)] and VDR positive [VDR(+)] sub-populations (i.e., VDR(+) flat, polygonal and adherent line; VDR(−) rounded, refractile and less adherent) [11]. For these experiments, SW480 cells were plated on cover slips and treated with or without 25(OH)D_3 (1 μM) for 24, 48 or 72 h. At the appropriate time points, cells were fixed and immunofluorescence was completed as described in Section 2. As shown in Fig. 2C, VDR expression levels were increased in the VDR(+) flat, polygonal and adherent line by 24 h post-treatment with 25(OH)D_3 and remained elevated 72 h after treatment. Cumulatively, these data show that 25(OH)D_3 treatment significantly increases the levels of 1α(OH)ase and VDR in colon cancer cells, and support the notion that 25(OH)D_3 can regulate the expression of VDR and 1α(OH)ase in a similar manner as the active metabolite *in vitro*. These results indicate that 25(OH)D_3 can exhibit similar anti-cancer activities as have been reported for 1α,25(OH)$_2$$D_3$.

3.2. The pro-hormone 25-hydroxyvitamin D3 exerts anti-proliferative effects on human colon cancer cells

The anti-proliferative effects of 25(OH)D_3 were investigating using the HT-29 cells. For these studies, cells were plated, allowed to adhere for 24 h prior to treatment with the different vitamin D compounds. As shown in Fig. 3, 5-day treatment of HT-29 cells with either 1α,25(OH)$_2$$D_3$ or 25(OH)D_3 significantly inhibited the growth of the HT-29 cells. The percent growth inhibition was 14, 65 and 53% for D3 (1 μM), 1α,25(OH)$_2$$D_3$ (0.5 μM) and 25(OH)D_3 (0.5 μM), respectively. A dose-dependent effect for 25(OH)D_3 treatment was also evident (53% versus 77%, for 0.5 and 1.0 μM) for the HT-29 cells. Similar findings were found in the Caco-2 and SW480 cells (data not shown).

In order to determine the effects of 25(OH)D_3 on cell cycle progression HT-29, Caco-2 and HCT116 cells were treated with 25(OH)D_3 (1 μM), 1α,25(OH)$_2$$D_3$ (0.5 μM) or ETOH for 48 h and DNA content was analyzed by flow cytometry. Our studies failed to reveal cell cycle arrest with either 1α,25(OH)$_2$$D_3$ or 25(OH)D_3 during the 48 h treatment period. However, using the tunnel assay, we found evidence of SW480 cells undergoing apoptosis following 72 h of treatment with either 1α,25(OH)$_2$$D_3$ (0.5 μM) or 25(OH)D_3 (1.0 μM). These results suggest that 25(OH)D_3 treatment may be inducing growth inhibition in part by causing apoptosis in the colon cancer cells.

4. Discussion

Vitamin D3 is biologically inert and requires successive hydroxylations by mitochondrial P450 enzymes present in the liver and kidney to form the biologically active hormone 1α,25(OH)$_2$$D_3$. The final step in metabolism is the conversion of pro-hormone 25(OH)D_3 to the active metabolite and hormone 1α,25(OH)$_2$$D_3$ by 1α(OH)ase. Due to

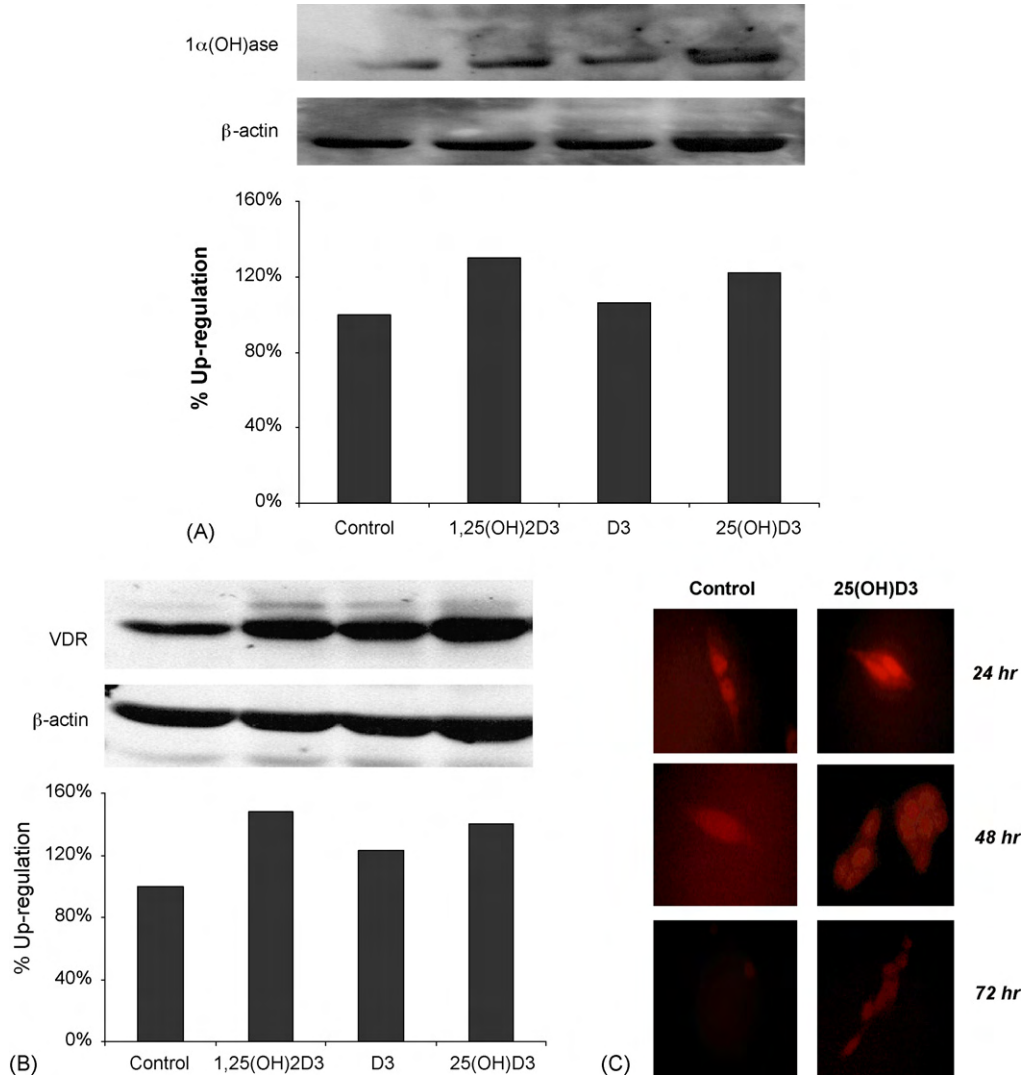

Fig. 2. Expression levels of 1α-hydroxylase and VDR in colon cancer cells treated with 25-hydroxyvitamin D3. Western blot analysis showing 1α-hydroxylase (A) and VDR (B) with appropriate actin control. HT-29 cells were treated with 1α,25(OH)$_2$D$_3$ (0.5 μM), D3 (1 μM), 25(OH)D$_3$ (1.0 μM) for 24 h. Both 1α,25(OH)$_2$D$_3$ and 25(OH)D$_3$ were found to up-regulate 1α-hydroxylase and VDR. (C) SW480 cells were plated and treated with control or 25(OH)$_3$ (1.0 μM) for 24–72 h. Immunofluorescence revealed an up-regulation of VDR by 24 h and show evidence that it remains elevated at 72 h following start of treatment.

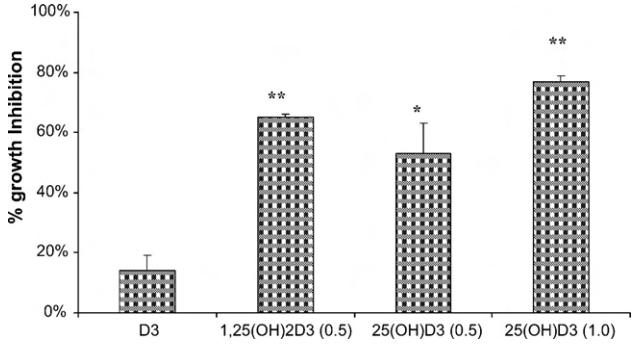

Fig. 3. HT-29 cells were treated with ETOH (control); cholecalciferol (negative control [1 μM]); 1,25(OH)$_2$D$_3$ (positive control [0.5 μM]); 25(OH)D$_3$ at 0.5 or 1.0 μM and cell number determined 5 days post-treatment. Percent growth inhibition is presented.

the hypercalcemic nature of 1α,25(OH)$_2$D$_3$, many relatively non-calcemic analogs of vitamin D have been synthesized and evaluated for their efficacy as anti-cancer agents. Ideally, the pro-hormone 25(OH)D$_3$, which is naturally occurring and non-toxic could be developed, however, due to the traditional concept of renal hydroxylation of the pro-hormone, until recently enthusiasm for establishing it as a cancer chemo-protective agent had been non-significant. In recent years, the expression of 1α(OH)ase, the enzyme that catalyzes this conversion, has been reported in several extra-renal tissues, including the pancreas, prostate and colon [12] leading to the hypothesis that 25(OH)D$_3$, may possess chemopreventive efficacy in the organs expressing endogenous 1α(OH)ase. In the colon, it has been reported that 1α(OH)ase is present in normal, and malignant colon tumors [3,4]. Results also showed that high- to moderately differentiated colon tumors express increased 1α(OH)ase, while poorly differentiated

tumors consistently have shown reduced expression or complete loss of 1α(OH)ase activity [5]. More recently, we have demonstrated that 1α(OH)ase and VDR are also highly expressed in the pre-cancerous lesions (ACF) of human colon samples [10]. Anti-proliferative effects of vitamin D analogs in colon cancer cells have been reported [13]. In this report, we evaluated effects of 25(OH)D_3 in several human colon cancer cell lines. Interestingly 25(OH)D_3 enhanced expression of both VDR and 1α(OH)ase in these cells. It is not clear whether 25(OH)D_3 directly suppresses cell proliferation of colon cancer cells *in vitro* or even if its conversion to 1α,25$(OH)_2D_3$ is required for its anti-proliferative actions. Nonetheless, these results provide the basis for further evaluation of 25(OH)D_3 as a candidate agent for the treatment of colon cancer. Furthermore, these findings support the possible therapeutic use of 25(OH)D_3 in individuals who are at high risk for developing colon cancer, including those with prior history of colonic polyps or with large numbers of ACFs.

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